STUDIES ON HUMAN K-CELL HAEMOLYSIS

by

STANISLAW JOSEPH URBANIAK B.Sc., M.B.Ch.B., MRCP(UK)

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TO ANN

Declaration

I confirm that the work in this thesis was conceived, planned and either executed by myself and/or by a technical assistant working directly under my supervision. Certain experiments were carried out in collaboration with other workers, and these are acknowledged in the text, as are certain procedures carried out on my behalf by other workers.

Signed ....

Date .....1/12/77.....

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SUMMARY

An in vitro homologous antibody-dependent cell-mediated cytolytic (ADCC) assay system was developed using human peripheral blood mononuclear cells, blood group O Rhesus (D) positive red blood cells and anti-D antibodies.

Extensive studies were carried out using monocyte-depleted cultures, where the lymphoid K-cell is the putative cytolytic effector cell, and the degree of specific lysis estimated from ^{51}Cr release from damaged RBC. Enhanced lysis was obtained when RBC were treated with the proteolytic enzyme papain. The coefficients of variation of replicates processed in an identical manner were less than 10%.
The degree of specific lysis of anti-D sensitised D-positive RBC depended upon the number of effector cells present, the number of RBC, and the amount of antibody. Under appropriate conditions, lysis was observed with only 3 ng anti-D per culture.

The specificity of lysis resided with the appropriate combination of antibody and antigen (ie. D-anti-D) and not with the effector cells. Donors of all ABO Rh groups were equally effective donors of K-cells and under appropriate conditions autologous RBC were lysed. The degree of specific lysis was influenced by the D-antigen dosage of RBC and the potential for determining the D-zygosity of RBC was explored.

Time-course experiments showed that RBC lysis was measurable by 30 minutes incubation, and was approximately linear over 18 hrs. at which time maximum lysis was seen. Cell-to-cell contact was essential for lysis and the cytolytic process was extremely short-range with no "innocent bystander"

effect being seen. Phagocytosis was not necessary for cytolysis to be observed, but effector cell mobility and intact microfilament function was required (inhibition by cytochalasin B). The divalent cations Ca^{2+} and Mg^{2+} were required for efficient RBC lysis. Studies with metabolic inhibitors showed that a metabolically active cell is required for K-cell activity, that both RNA and protein synthesis are necessary for maximum lysis, and that an intact microtubule system is also required.

The trigger to lysis appears to be the activated Fc component of red cell-bound anti-D which interacts with the K-cell Fc receptor. Lysis was mediated by IgG_1 antibodies and was significantly inhibited only by IgG_1 or IgG_3 immunoglobulins, not by IgG_2, IgG_4, IgA or IgM. This suggests that the K-cell Fc receptor is specific for IgG but that IgG_1 and IgG_3 cross-react.

The K-cell has the morphologic appearance of a small lymphoid cell, does not appear to have surface immunoglobulin or sheep RBC (E) receptors but does have Fc and C3 receptors. The cell is non-adherent, non-phagocytic and negative for the non-specific esterase stain. It therefore does not have any of the characteristics of a mature monocyte, but does have monocyte-specific surface antigens as demonstrated by anti-monocyte serum. It may therefore be a monocyte precursor.

Application of the K-cell assay to normal individuals revealed that there was inherent biological variation between individuals and that there appeared to be 'good' and 'poor' K-cell donors in terms of cytolytic activity. K-cells were present in the cord blood of neonates and were slightly less active than adult cells. Adrenalin infusion produced a transient rise in K-cell activity at 15-30 minutes and infusion of D

positive RBC into a D negative individual resulted in a transient fall at 12-14 days followed by enhanced lytic potential some weeks later. Preliminary studies into the biological activities of different sources of anti-D revealed that there was poor correlation between agglutination titre and specific RBC lysis. The potential applications of the K-cell assay system are discussed.

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1.0 HUMAN HAEMOLYTIC DISEASE

1.1 Introduction

A number of disease states, whether congenital or acquired, have a haemolytic component ie. the destruction of erythrocytes is involved. In some cases the disease process directly involves the haemopoietic tissue, in others, the RBC is an "innocent bystander". It is possible to classify these haemolytic conditions in a variety of ways and a simplified version is shown in table 1.1.1.

Of particular interest are the haemolytic conditions mediated by antibodies since these are often encountered in Blood Transfusion practice. The mechanism of lysis of red cells is basically the same whether mediated by transfused alloantibodies as in a haemolytic transfusion reaction, by transplacental transfer of alloantibodies as in haemolytic disease of the newborn, or whether mediated by auto-antibodies. The mechanisms whereby antibody-coated RBC are destroyed are detailed in table 1.1.2.

1.2 Antibody-mediated RBC destruction

There is a great deal of evidence that the attachment of antibody to red cell surface antigens does not cause damage *per se* (Mollison 1972) but that this follows either as a result of activation of the complement system or interaction with mononuclear phagocytes in the reticulo-endothelial system (RES) of the liver and spleen. The ability of particular antibodies to cause RBC destruction depends upon their thermal range, concentration, immunoglobulin class and sub-class and whether or not they activate complement. To some

Table 1.1.1 HAEMOLYTIC CONDITIONS IN MAN

A. Intrinsic defects of the erythrocyte (inherited):

- abnormal membrane (eg. hereditary spherocytosis)
- abnormal haemoglobin (eg. sickle-cell disease)
- abnormal intracellular enzymes (eg. pyruvate kinase deficiency)

B. Acquired defects:

1) non-immunological

- mechanical trauma (eg. heart valves)
- physical trauma (eg. burns)
- infectious agents (eg. malaria)
- chemicals, drugs, and venoms (eg. viper venom)

2) immunological (ie. antibody mediated destruction)

a) alloantibody mediated

- transfusion reactions
- haemolytic disease of the newborn
- certain drugs (eg. penicillin)

b) autoantibody mediated

- idiopathic - "warm"
  "cold"
- symptomatic (eg. S.L.E.)
- certain drugs (eg. methyl dopa)

Table 1.1.2 ANTIBODY-MEDIATED RBC DESTRUCTION

1) RBC + antibody (IgM) → C1 - C9 complement activation → intravascular lysis

2) RBC + antibody (IgG or IgM) → complement activation to C3 → removal by R.E.S. (mainly liver)

3) RBC + antibody (IgG) → directly removed by R.E.S. (mainly spleen)

4) RBC + antibody (IgG) → antibody dependent cell mediated lysis

extent, the clinical manifestations of haemolysis reflect the method and rapidity of destruction and this in turn is dependent on the type of antibody. Those antibodies which "fix" complement and have a thermal range which includes activity at a body temperature ($37^{o}C$) in general cause rapid intravascular lysis of red cells (mechanism (1) table 1.1.2). The red cell membrane is damaged as a result of activation of the complement components C1 to 9 with "holes" being produced by enzymic action followed by osmotic lysis as a result of ion fluxes and water entering the RBC (Muller-Eberhard 1969). These antibodies are usually of the IgM class and the most important clinically are the isohaemagglutinins of the ABO blood group system, anti-A and anti-B. Autoimmune haemolytic anaemia can also result from IgM antibodies activating complement - usually cold agglutinin disease where the antibodies are also active at body temperature. Occasionally IgG antibodies will cause rapid intravascular lysis by direct complement activation - the best known being the Donath-Landsteiner antibodies of paroxysmal cold haemoglobinuria. "Immune" IgG anti-A or anti-B can also activate complement to cause intravascular lysis. The complement system includes a series of inactivators which prevent a biological reaction from becoming pathological, and one of the most important is the C3b inactivator. When a red cell bound antibody activates complement, C3b is generated on the RBC membrane and this then results in activation of the terminal complement components to produce lysis. The C3b inactivator acts rapidly to cleave C3b to an inactive component C3d and so intravascular

haemolysis will not occur if the deposition of C3b is slow or small in amount. For example, in cold agglutinin disease a dynamic equilibrium may be reached where the majority of the RBC are coated with C3d and are then resistant to further haemolysis. In so-called "warm antibody" haemolytic anaemia with IgG auto-antibodies, complement deposition can also be demonstrated on the RBC, although direct complement-mediated lysis is rarely seen.

It has been shown that complement coated RBC, with or without bound IgG, are bound by mononuclear phagocytes of the reticulo-endothelial system (RES) (Abramson et al 1970a) by means of cell-surface receptors for C3b/C3d and IgG. This process has been called "immune-adherence" and is usually a prelude to phagocytosis and intracellular destruction of RBC or micro-organisms. It has also been shown in vitro that the binding of the RBC to monocytes/macrophages results in distortion of the RBC and fragments may be "bitten off" resulting in spherocytes and RBC fragments (Lobuglio et al 1967; Abramson et al 1970b). This mechanism has been postulated to result in the in vivo blood picture of spherocytosis, RBC fragmentation and intravascular haemoglobin seen in immune haemolytic anaemia. By the use of ^{51}Cr-labelled RBC it has been shown that C3 coated RBC are removed more efficiently by the liver than by the spleen (Cooksley et al 1973) since the liver has a larger blood supply. This type of RBC destruction has been termed "extravascular lysis" and is thought to be the main mechanism of removal of RBC coated with non-complement fixing IgG antibodies.

However, the binding and phagocytosis of sensitised RBC by monocytes is inhibited by microgram quantities of normal IgG and therefore should be completely inhibited by

the IgG concentration in normal plasma *in vivo*. It has been suggested that the micro-environment of the spleen may be more akin to the *in vitro* situation with haemoconcentration, very little plasma and a great excess of mononuclear cells which would tend to favour adherence and phagocytosis of sensitised RBC. Furthermore, the amount of IgG antibody required for immune adherence, in the absence of complement, is quite considerable (approximately 100 times more - Ehlenberger & Nussenzweig 1977).

In recent years investigations have demonstrated that extracellular lysis of RBC can take place without the necessity for phagocytosis and that very small amounts of antibody are required (Holm 1972). It is possible that this is a more important mechanism *in vivo* than immune adherence especially where antibody coating of RBC is minimal. It may also be an important mechanism for the lysis of RBC coated with non-complement fixing antibodies such as those of the Rhesus blood group system.

It has been shown that IgG antibodies such as anti-D (Vogel et al 1943), anti-Kell (Swisher & Young 1954) and anti-Duffy^{a} (Wiener 1954) which are thought to mediate "extravascular" lysis in the spleen very occasionally cause rapid RBC destruction with substantial intravascular haemolysis resulting in haemoglobinuria. It has been suggested that this is due to the rapid formation and lysis of spherocytes by immune adherence but it is equally possible that extracellular lysis is the cause.

This type of lysis has been described as antibody-dependent cell-mediated lysis (ADCC) and is a mechanism which has been shown to result in damage to many types of cells

including allogeneic and xenogeneic RBC, tumour cells, and lymphocytes, and is described in some detail in the following section.

2.0 ANTIBODY DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC)

2.1 Introduction

The cytotoxic action of normal lymphoid cells against antibody-coated target cells was first observed by Moller (1965) and described in detail by Perlmann & Holm (1969) and MacLennan et al (1969). Perlmann and colleagues used a system consisting of purified human lymphoid cells "attacking" chicken RBC sensitised with a rabbit anti-chicken RBC (Perlmann et al 1972). MacLennan and colleagues used a system consisting of rat lymph node cells and human lymphoid cells "attacking" a human liver cell-line (Chang) sensitised with rabbit anti-Chang antibodies (MacLennan 1972). Despite such bizarre species combinations, a number of common features were observed which distinguished the mechanism from the classical direct T-lymphocyte cytotoxicity described by Cerottini et al (1970).

(1) the effector cell did not require prior immunisation against the target cell antigens.

(2) the T-lymphocyte was not the effector cell.

(3) antibody was required for the induction of lysis.

It was further demonstrated that the role of antibody was quite different from that in complement-mediated lysis in that the ADCC system was complement-independent. This was later confirmed by van Boxel et al (1974) who also excluded the possibility of endogenous synthesis of complement components during the incubation period.

The essential requirements for the above antibody-mediated lysis are: (1) a suitable source of effector cells, (2) appropriate sensitising antibody and (3) a suitable target cell.

2.2 The nature of the effector and target cell - influence on ADCC

In the original systems described (Perlmann et al 1972) (MacLennan 1972) purified human or rat "lymphocytes" were used. The situation has become greatly complicated however because of differences in definition and terminology, source and treatment of the effector cells, and possible species differences.

For example, the lytic mechanism has been described as antibody-dependent cell-mediated cytotoxicity (ADCC and Ab-CMC by different authors), antibody dependent lymphocyte cytotoxicity (ADL), antibody-dependent cell-mediated immune lympholysis (ABCIL), antibody-dependent lymphocyte-mediated cytotoxicity (Ab-LMC) antibody mediated cell cytotoxicity (AbMC) lymphocyte-dependent antibody cytotoxicity (LDAC), or quite simply K-cell cytotoxicity. Since the consensus abbreviation is ADCC I shall use this to indicate the general phenomenon, and "K-cell cytotoxicity" for non-monocytic lysis (see below).

Mononuclear lymphoid cells from numerous species have been shown to be effective in ADCC and these are shown in table 2.2.1. The great variety of target cells used in various studies is shown in table 2.2.2. Since the subject of this thesis is concerned only with human work, animal experiments will only be quoted where the relevant human information is unavailable, since there are indications of important species variations with different ADCC systems (Zighelboim & Gale 1974).

It has been shown that human cells other than purified lymphoid cells are active in ADCC, such as polymorphonuclear

phagocytes (Gale & Zighelboim 1974) and monocytes/macrophages (Holm 1972). These cells are known to participate in immune-adherence, phagocytosis and intracellular killing of micro-organisms and erythrocytes coated with antibody, with or without complement, and have the appropriate receptors for the Fc part of immunoglobulins and complement C3 (reviewed by Stossel 1975).

Great interest has been shown in the true identity of the cell with lymphocyte morphology which mediates ADCC as originally described by Perlmann and MacLennan. A great deal of the earlier confusion in the literature was due to the failure to appreciate the importance of the nature of the target cells and antibody, (see below) and that there might be inter-species differences.

An important observation was that the type of target and source of antibody are important in determining the nature of the effector-cell mediating lysis. The available evidence for the human mononuclear effector cell differences is summarised in table 2.2.3. In general it appears that highly purified lymphoid cells are much more effective in promoting the lysis of nucleated target cells such as allogeneic lymphocytes and tumour cell lines whereas monocytes are more effective in lysing RBC targets. It is interesting to note that chicken RBC are nucleated and appear to be equally susceptible to lysis by monocytes and lymphoid cells. As yet, there is no explanation of these interesting and important differences.

The nature of the non-monocytic lymphoid cell is a matter of confusion and terms such as cytotoxic lymphocytes,

cytotoxic B cells, "null" cells, A-cells and K-cells have used to describe essentially the same sub-population of cells. The confusion is mainly caused by the overlap in morphological features and surface membrane receptors which have been used to identify monocytes and the T and B lymphocyte sub-populations. The surface marker methods have been extensively reviewed (Transplantation Reviews 1973, vol 16 - many authors). The consensus opinion is that T lymphocytes may be identified by the sheep RBC (E) receptor and B lymphocytes by surface immunoglobulin (SIg) formed by endogenous synthesis. B lymphocytes (or a major sub-population) possess receptors for the Fc part of IgG, but so do monocytes and it also appears that a minor sub-population of T lymphocytes have Fc receptors (mainly for IgM). The complement C3 receptor is found on all monocytes, the majority of B lymphocytes, and a minor population of T lymphocytes.

By the above methods the lymphoid effector cell (K-cell) has been identified as a minority population of cells and the only essential surface receptor is that for the Fc part of IgG (see table 2.3.1). However, not all Fc-bearing cells are necessarily K-cells. The consensus view is that the K-cell is not a T lymphocyte (see table 2.2.4) and that it possesses a C3 receptor (see table 2.2.5). The presence of an SIg receptor is more contentious (see table 2.2.6) and controversy may have been resolved by the findings of Brier et al (1975) and MacDermott et al (1975) that the major population is SIg negative but that a SIg positive population is also effective, and they propose that the K-cell belongs to the B-lymphocyte series, possibly as a precursor of the readily identifiable SIg positive B-cell.

Table 2.2.1 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

Some species where effector cells have been demonstrated

Human	-	Perlmann & Holm (1969)
Rats	-	MacLennan et al (1969)
Mice	-	van Boxel et al (1972)
Ducks	-	Bubenik et al (1970)
Rabbits	-	Bona et al (1975)
Guinea-pigs	-	Hunninghake & Fauci (1976)

Table 2.2.2 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

Some examples of various target cells used:

Erythrocytes

Human - Holm (1972)

Chicken - Perlmann & Holm (1969)

Sheep - Calder et al (1974)

Duck - Bubenik et al (1970)

Donkey - van Boxel et al (1974)

Human lymphocytes - Hersey et al (1973)

Human liver cell line (Chang) - MacLennan (1972)

Human lymphoblastoid cell line (RPMI-4265) - Zighelboim & Gale (1974)

Human fibroblasts - Moller & Svehag (1972)

Mouse EL4 leukaemia cell line - Zighelboim & Gale (1974)

Moloney-virus induced mouse cell line - Lamon et al (1977)

Mouse mastocytoma (P815 - X2) - MacDonald & Bonnard (1975)

Sheep fibroblasts - Moller & Svehag (1972)

Rat thymocytes - Strom et al (1975)

Table 2.2.3 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

Human mononuclear effector cells - restrictions in mediating lysis due to the nature of the target cell

1) Purified human lymphoid cells* effectively lyse sensitised lymphocytes

Hersey et al (1973)

Kovithavongs et al (1975)

Rachelefsky et al (1975)

Werner et al (1976)

2) Human monocytic cells are ineffective in lysing sensitised allogeneic lymphocytes

Kovithavongs et al (1975)

3) Purified human lymphoid cells effectively lyse sensitised transformed cell lines

MacLennan (1972) - Chang liver cells

Zighelboim et al (1974) - mouse EL4 cells

Zeijlemaker et al (1975) - mouse mastocytoma cells

4) Human monocytes are ineffective in lysing sensitised transformed cell lines

Zeijlemaker et al (1975)

5) Purified human lymphoid cells effectively lyse sensitised chicken RBC

Perlmann et al (1972)

Wisløff et al (1974 a & b)

Calder et al (1974)

6) Human monocytes effectively lyse sensitised chicken RBC

Poplack et al (1976)

Table 2.2.3 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

(contd.)

7) Purified human lymphoid cells are ineffective in lysing sensitised human RBC

Holm (1972); anti-A, B and D against group A or B or Rhesus (D) pos. RBC

Kovithavongs et al (1975); anti-A or B against group A or B RBC

Zeijlemaker et al (1975); rabbit anti-HRBC against group O RBC

8) Purified human lymphoid cells effectively lyse sensitised human RBC

Hinz & Chickosky (1972); anti-A and anti-D against group A or rhesus (D) positive RBC

9) Human monocytes effectively lyse sensitised human RBC

Holm (1972); details as above

Holm et al (1974); details as above

Kovithavongs et al (1975); details as above

Zeijlemaker et al (1975); details as above

Poplack et al (1976); anti-B with group B RBC

* cells with the morphological appearance of lymphocytes with no adherent or phagocytic properties.

Table 2.2.4 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

Evidence that the human lymphoid effector cell is not a T-lymphocyte

Brier et al (1975)

- E-rosette enriched populations showed poor ADCC
- E-rosette depleted populations showed enhanced ADCC

Zighelboim et al (1974)

- thymocytes obtained at operation were inactive in ADCC
- E-rosette enriched populations were inactive in ADCC

MacDermott et al (1975)

- E-rosette enriched populations were inactive in ADCC
- E-rosette depleted populations showed enhanced ADCC

Zeijlemaker et al (1976)

- E-rosetting cells associated with poor ADCC

Wisløff et al (1974 a)

- E-rosette depletion enhances ADCC

Rachelefsky et al (1975)

- Immunodeficiency patients with isolated T cell defects had normal ADCC

Table 2.2.5: ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

Evidence that the human lymphoid cell has complement C3 receptors

Brier et al (1975)

- removal of EAC3 rosette-forming cells abolished ADCC

Perlmann et al (1975)

- removal of EAC3 rosette-forming cells abolished ADCC
- passage down human C3b columns diminished ADCC

Wahlin et al (1976)

- demonstration that 50% of plaque-forming cells on IgG-EA monolayers form EAC3 rosettes

Yust et al (1975)

- indirect evidence that cells cytotoxic to target monolayers were EAC3 rosette-forming cells.

Table 2.2.6 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

Evidence for and against the presence of surface-membrane immunoglobulin on the human lymphoid effector cell

a) that SIg is not present

Brier et al (1975)

- removal of SIg+ cells on F(ab)2 anti-human immunoglobulin columns did not reduce ADCC
- the enriched SIg+ cells showed variable but low ADCC

MacDermott et al (1975)

- removal of SIg+ cells on anti-immunoglobulin columns did not reduce ADCC, but SIg+ cells also demonstrated ADCC

Wahlin et al (1976)

- demonstrated some SIg+ cells on plaques but that these were T cells with adsorbed Ig

Rachelefsky et al (1975)

- a variety of immunodeficiency patients studied showed strong correlation between ADCC and SIg+ cells

Zeijlemaker et al (1976)

- removal of SIg+ cells by nylon wool adherence did not reduce ADCC
- cells adherent to EA-monolayers were depleted of SIg+ cells but demonstrate enhanced ADCC

Calder et al (1974)

- removal of SIg+ cells on anti-immunoglobulin columns did not reduce ADCC

Wisløff & Froland (1973)

- removal of SIg+ cells by nylon wool adherence did not reduce ADCC
- B cell deficient hypogammaglobulinaemics have normal ADCC

Table 2.2.6 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

Evidence for and against the presence of surface-membrane immunoglobulin on the human lymphoid effector cell (contd)

b) that SIg is present

MacDermott et al (1975)

- removal of SIg+ cells on anti-immunoglobulin columns did not reduce ADCC, but SIg+ cells also demonstrated ADCC

Rachelefsky et al (1975)

- a variety of immunodeficiency patients studied showed strong correlation between ADCC and SIg+ cells

Yust et al (1975)

- indirect evidence that cells cytotoxic to monolayers had SIg receptors.

2.3 Antibody requirements for ADCC

It has been conclusively demonstrated that antibodies of the IgG class are required for the induction of lysis by the K-cell (table 2.3.1) and also by the monocyte (Holm et al 1974) and that the Fc part of the molecule attaches to the Fc receptor on the effector cells (table 2.3.1). Enzyme digestion studies indicate that both the CH3 and CH2 domains on the heavy chain Fc part are required for efficient lysis (MacLennan et al 1974; Michaelson et al 1975).

Immunoglobulins of the IgG_1 and IgG_3 subclasses are the most efficient at inducing target cell lysis (Larsson et al 1975; Holm et al 1974) as well as in inhibiting antibody-mediated lysis (see table 2.2.2).

From the subtle differences in inhibition pattern with the lymphoid K-cell and monocyte effectors it would appear that these cells may have different Fc receptors and this may in part explain the differences in target lysis as noted above. The Fc receptors on both cell types appear to be relatively "broad-spectrum" in that IgG_2 subclass target cell antibodies can be inhibited by IgG_1 and IgG_3 in monocyte lysis, and by IgG_1, IgG_2 and IgG_3 in K-cell lysis (Larsson et al 1975).

Furthermore, for the monocyte, <u>both</u> IgG_1 and IgG_3 have been shown to inhibit target cell lysis mediated by <u>either</u> IgG_1 or IgG_3 subclass antibodies indicating cross-reactivity of the Fc receptor for IgG_1 and IgG_3 (Holm et al 1974). This conclusion has also been reached for monocyte immune-adherence (Abramson & Schur 1972).

The Fc receptors on the effector cells are also triggered by IgG from a variety of different animal species indicating a lack of specificity, although certain restrictions may be

seen (Zighelboim & Gale 1974).

The amount of antibody required to sensitise target cells is very small. Under standard conditions, 50% lysis of chicken RBC can be obtained with as little as 0.1-1.0 ng of IgG antibody (Perlmann et al 1972). This amount is too low to activate complement and indicates the great sensitivity of ADCC. Furthermore the demonstration of ADCC activation by non-complement fixing antibodies (IgG_2 - Larsson et al (1976); Rhesus blood group antibodies - Holm et al (1974) increases the potential biological spectrum of activity of such antibodies in immune mechanisms.

It has been shown that the lytic process is independent of complement activation (van Boxel et al 1974) in vitro, but it is possible that there is co-operation between antibody and complement in vivo to make the binding of effector to target cell more efficient since target-bound complement is itself unable to induce cytotoxicity, but enhances cytotoxicity at low antibody concentrations (Perlmann et al 1975; Lustig & Bianco 1976).

2.4 Mechanism of lysis

A great deal of information on the extracellular killing of target cells has been obtained from studies of T lymphocyte toxicity (Cerottini & Brunner 1974) and in animal models there appear to be many similarities in the mechanism of lysis of T-cell cytotoxicity and ADCC cytotoxicity (Strom et al 1975). Evidence is also accumulating that the same appertains to the human effectors in both cytotoxic systems (MacDonald & Bonnard 1975).

In animal models the lytic mechanism has been shown to take place in three separate stages: (1) an initial

Table 2.3.1 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

Evidence that the human lymphoid effector cell interacts with the Fc part of IgG target cell antibody to induce lysis

1) F(ab)2 fragment of anti-target IgG antibody fails to induce ADCC.

Moller & Svehag (1972)
Larsson & Perlmann (1972)
MacLennan et al (1973)

2) IgM target antibodies are ineffective in ADCC*.

MacLennan (1972)
Perlmann et al (1972)

3) Normal IgM, IgA, IgD and IgE are not inhibitory to ADCC.

Wisløff et al (1974b)

4) Excess normal IgG, but not the F(ab)2 fragment, inhibits ADCC.

Wisløff et al (1974b)
Larsson et al (1973)

5) Isolated Fc fragments of normal IgG inhibit ADCC.

Wisløff et al (1974b)

6) Aggregated IgG in excess inhibits ADCC.

MacLennan (1972)
Perlmann et al (1972)

Table 2.3.1 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

Evidence that the human lymphoid effector cell interacts with the Fc part of IgG target cell antibody to induce lysis (contd)

7) Antigen-antibody complexes (in excess antigen) inhibit ADCC.

MacLennan (1972)

Perlmann et al (1972)

8) Passage down an antigen-antibody column decreases ADCC.

Zighelboim et al (1975)

Wisløff et al (1974a)

9) Depletion of EA-rosette-forming cells decreases ADCC and vice versa.

Wisløff et al (1974a)

Hallberg (1974)

10) Depletion of Fc bearing cells by EA monolayers decreases ADCC and vice versa.

Zeijlemaker et al (1976)

* recent evidence suggests that in mice (Lamon et al 1977) and humans (Wahlin et al 1976) IgM may induce cytotoxicity, but by a T-lymphocyte effector and not a K-cell.

Table 2.3.2 : ANTIBODY-DEPENDENT CELL-MEDIATED CYTOXICITY

IgG subclass inhibition of ADCC by K-cells and monocytes

	ADCC mediated by effector	
	K-cell	monocyte
native IgG_1	inhibits	inhibits
IgG_2	inhibits poorly	no inhibition
IgG_3	inhibits	inhibits
IgG_4	no inhibition	no inhibition
aggregated		
IgG_1	inhibits	inhibits
IgG_2	inhibits	inhibits poorly
IgG_3	inhibits	inhibits
IgG_4	no inhibition	no inhibition

data from Larsson et al (1973) and (1975); MacLennan et al (1973) and Holm et al (1974)

recognition stage where the effector cell surface Fc receptors interact with IgG complexed to the target cell antigens, (2) a post recognition 'hit' where the target cell membrane is damaged irreversibly in some way, and (3) target cell disintegration which does not require the continued presence of the effector cell (reviewed by Cerottini & Brunner 1974). The killing process can be very rapid with complete lysis of targets within a few hours. The initial recognition stage involves direct cell-cell contact (Perlmann and Perlmann 1970). Divalent cations are required for T cell recognition but not for K-cell recognition (Goldstein & Smith 1976). It is relevant to note that divalent cations are not required for the binding of IgG coated RBC in rosette tests (Lay & Nüssenzweig 1969). There are further differences in the cation requirements during the lethal hit stage in that Ca^{2+} is required for T-cell cytotoxicity and Mg^{2+} for K-cell cytotoxicity. The situation is further complicated by the fact that the divalent cation requirements for ADCC of RBC and nucleated targets differ (Goldstein & Fewtrell 1975) in that RBC lysis was shown to be only partially Mg^{2+} dependent whereas Chang cell lysis required Ca^{2+}. The nature of the lytic process is unknown but studies with intracellular markers of various sizes suggests that lysis is osmotic following the influx of cations and water through the damaged target cell membrane (Henney 1973).

Time lapse photography has shown that cytotoxic T lymphocytes are able to move from target to target (Koren et al 1973) and a similar phenomenon has been reported for the lysis of RBC on monolayers by K-cells (Biberfield et al 1975). This is in keeping with the findings that continued contact of effector cell and target cell is not necessary for eventual lysis. Furthermore, it can be calculated from

estimates of numbers of K-cells in culture and the numbers of target cells lysed, that more than one target must be lysed per effector (MacLennan 1972; Perlmann et al 1972).

There is insufficient evidence at present to indicate whether the extracellular lytic mechanism is fundamentally the same for all mononuclear cell effector types, or whether there are differences which would in part explain the preferential lysis of certain target cell types.

2.5 Clinical relevance of ADCC

2.5.1 Role in immunity to infection

Antibody plays a complex role in the defence against infection in that a number of possible mechanisms operate. Complement-fixing antibodies may activate complement by the "classical pathway", ending with lysis of the organism. Coating of micro-organisms with antibody with or without complement (ie. opsonisation) will allow phagocytic polymorphs or monocyte/macrophages to adhere, phagocytose and ingest these particles. An alternative possibility is that antibody may co-operate with certain effector cells to mediate ADCC resulting in extracellular as opposed to intracellular killing. One could postulate that this mechanism is complementary to that of phagocytosis in that the amount of target bound antibody may be insufficient to activate C3 but sufficient to activate ADCC. Alternatively, the type of antibody may bind complement poorly, or the antigen distribution on the target cell may be too low to activate complement yet sufficient to activate ADCC.

A variety of organisms have been shown to be susceptible

to ADCC including meningococci (Lowell et al 1976); schistosomula or schistosoma mansoni (Dean et al 1974) cryptococcus neoformans (Diamond 1974) and herpes virus infected cells (Rager-Zisman et al 1974) indicating that ADCC may be a mechanism of general biological importance.

2.5.2 Role in tumour immunity

As indicated by the variety of target cells used, the possible role of ADCC in defence against tumours has received considerable attention in recent years. There is little doubt that in vitro, mononuclear effector cells effectively lyse appropriately sensitised tumour cell lines. This provides a rational basis for the co-operation of antibody and immunocytes in the elimination of certain tumours. The demonstration of both T-cell cytotoxicity and K-cell cytotoxicity in tumour bearing mice has been extensively studied by Lamon et al who have shown that an already complex situation is even more so (Lamon et al 1977). Evidence is presented for the participation of K-cells and IgG antibodies in classical ADCC, for T lymphocytes mediating the lysis of tumour cells sensitised by IgM antibodies and for T lymphocytes lysing tumour cells directly, without the participation of antibody.

The demonstration that antigen-antibody complexes can inhibit ADCC (MacLennan 1972) suggests that a possible defence mechanism of growing tumours is the shedding of surface antigen complexed with antibody, which may then inhibit cell-mediated lysis. Indirect evidence has been presented by Hersey et al (1976) who showed that plasma exchanges restored ADCC activity in melanoma patients who had been shown to have serum blocking factors. It has also been demonstrated that

lymphoid cells from normal mice acquire tumour-specific ADCC when injected with serum from tumour-bearing mice (Pollack 1973). This has been interpreted as showing that immune-complexes (consisting of target cell antibody and soluble tumour antigen in antibody excess) will bind to K-cells and the free antibody valencies are then able to bind to the target cell.

The ability to "arm" mouse effector cells with immune complexes has also been shown with chicken RBC (Greenberg & Shen 1973).

There is thus sufficient evidence to suggest that ADCC may be an important mechanism for tumour immunity, but the interaction of the various defence mechanisms *in vivo* is unknown at present.

2.5.3 Role in Allograft rejection

The major cell involved in graft rejection is the T lymphocyte (Cerottini & Brunner 1974) and it is known that preformed antibody can result in hyperacute rejection (Kissmeyer-Nielsen et al 1966), possibly as a result of complement activation and platelet aggregation, and in accelerated rejection if formed after transplantation (Mittal et al 1975). The incidence of such rejections has been reduced by the detection of preformed antibodies in a complement-dependent cytotoxic system directed against lymphocytes. It has been recently demonstrated however, that certain antibodies can only be detected by ADCC involving lymphocytes (Ting & Terasaki 1975) and that recipients negative for such antibodies have a better renal graft survival. *In vitro* evidence has also been presented for the lysis of donor lymphocytes by ADCC using serum from recipients of the donor's

kidney (d'Apice et al 1974).

There is therefore evidence that ADCC may play a role *in vivo* in rejection of tissue transplants, but the relative importance of this mechanism is unknown.

2.5.4 Role in autoimmune disease

ADCC mediated by lymphoid K-cells is effective in lysing thyroglobulin-coated RBC (Calder et al 1973) and it is possible that a similar mechanism operates *in vivo* in autoimmune thyroiditis resulting in thyroid damage.

Evidence has been presented that non-T lymphocytes from patients with chronic active hepatitis mediate lysis of isolated liver cells. It has not been conclusively shown that the mechanism is antibody-mediated but the evidence is suggestive (Cochrane et al 1976). In ulcerative colitis, local lymph node cells have been shown to be cytotoxic to foetal colon cells, although the exact nature of the antibody requirements has not been investigated (Perlmann & Broberger 1963). The evidence that ADCC is active in organ-specific autoimmune disorders is therefore rather poor. In generalised autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis, immune-complex formation is common and it is possible that these complexes interfere with ADCC activity to infections.

In autoimmune haemolytic anaemia where RBC are coated with IgG antibodies with or without complement, it is possible that ADCC is active, although this has not been formally demonstrated. It is known that monocyte adherence results in spherocytosis and phagocytosis and monocytes have been shown

(detailed above) to lyse allo-antibody coated human RBC by an extracellular mechanism and thus have the capacity to lyse auto-antibody-coated RBC. It is possible therefore that this is a method of clearing antibody-coated RBC when the IgG level is too low, or of an inappropriate subclass to activate complement.

CHAPTER II - AIMS OF THE STUDY

AIMS OF THE STUDY

The observations of Hintz & Chickosky (1972) suggested that purified human lymphoid cells were able to lyse anti-D coated RBC. However, the more detailed work of Holm and colleagues showed that monocytes were more active in this respect, and that anti-A was even better at inducing extra-cellular lysis of RBC (Holm & Hammarstrom 1973) (see chapt. I, table 2.2.3).

It was therefore decided to investigate the role of ADCC of human RBC in more detail, with particular emphasis on the role of anti-D. The study of an antibody of the Rhesus system is particularly relevant because:

1) apart from one exception (Ripley serum) Rhesus antibodies do not fix complement and the lytic effects of antibody alone could be studied.

2) second to the ABO system, the Rhesus system is clinically the most important in Transfusion practice in view of the immunogenicity of the antigens (in particular the D antigen).

3) Rhesus antibodies are the most important in producing haemolytic disease of the newborn (and anti-D was most often implicated until the development of the Rh. prevention programme).

4) in "warm" antibody autoimmune haemolytic anaemia, blood group specificity for the Rhesus system can often be shown, usually against an unidentified common antigenic determinant (but occasionally against the "e" antigen (Dacie 1975)).

The mechanism of *in vivo* lysis of allo and auto-antibodies cannot be predicted with any certainty by currently available *in vitro* tests, even on the basis of immunoglobulin class and complement binding activity. It would therefore be advantageous to develop such an

assay with a view to its application in the above conditions.

Assuming that an ADCC assay can be developed using anti-D, then this test should have wide application because it is now apparent that a homologous system is required for human work (Zighelboim & Gale 1974). Homologous systems using human lymphocytes sensitised with anti HL-A antibodies have been described (Hersey et al 1973) but suffer from the disadvantage that good antisera are not readily obtained and histocompatibility differences (or similarities) between the lymphoid effector and target cells may interfere with the assay. A D-anti-D system would be more acceptable due to the ready availability of anti-D and D-positive RBC.

In view of the conflicting literature reports on the role of the lymphoid K-cell in lysing human RBC in ADCC I decided to restrict investigations mainly to the use of monocyte-depleted cultures since monocyte ADCC had already been well documented (Holm 1972; Holm & Hammarström 1973).

Three main objectives were therefore set prior to the commencement of the present study;

1) to demonstrate that lymphoid K-cells are active in ADCC against human RBC.

2) to characterise the basic haemolytic reaction <u>in vitro</u> and to develop an ADCC assay on this basis.

3) to apply this information to the study of human haemolytic and related conditions.

CHAPTER III - MATERIALS AND METHODS

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1.0 PREPARATION OF MONONUCLEAR CELL SUSPENSIONS

Mononuclear cells were separated from heparinised venous blood (10 u/ml preservative free) as recommended by the I.U.I.S. (1975) by density gradient centrifugation over Ficoll-Triosil (F-T) specific gravity 1.077, (1 vol. whole blood to 1 vol. Ficoll-Triosil). Samples were centrifuged at 400 g for 40 minutes at room temperature, carefully removed from the centrifuge, the upper plasma layer containing mainly platelets discarded and the mononuclear cell layer at the interface of plasma-F-T harvested. The cell suspension was diluted to a convenient volume with tissue culture medium 199 (TC 199) (Gibco-Biocult) containing 25 mM/l HEPES and 100 u/ml penicillin G and 100 µg/ml streptomycin and centrifuged at 400 g for a further 20 minutes. The supernatant was discarded and the cell pellet washed x 2 in TC 199 by centrifuging at 200 g for 10 minutes. The cells were then finally resuspended in TC 199 supplemented with 10% Human group AB serum, and an aliquot removed to estimate the number of cells, and percentage monocytes. The mononuclear cell suspension was then used directly for surface marker studies or K-cell studies, or more usually, adherent monocytes were removed as described below. The viability of the cells was checked by Trypan Blue dye exclusion and was always >95% and usually >99%.

1.1 Preparation of Ficoll-Triosil

Solution A: 9% Ficoll

90 g Ficoll (M.W. 400,000; Pharmacia) are dissolved

in 1000 ml sterile distilled water kept at 37°C in a water bath.

Solution B: 33.9% Triosil

200 ml Triosil (Nyegaard & Co.) 440 are made up to 445 ml with sterile distilled water.

Mix 960 ml solution A with 400 ml solution B. Adjust specific gravity to 1.076 - 1.078 and distribute into Medical Flats (approximately 75 ml into each).

Autoclave for 10 minutes at 151 lb/square inch. Store in the dark at 4°C.

1.2 Lysis of contaminating red cells

In certain experiments eg. rosetting techniques (see below) and occasionally when there was residual red cell contamination of the mononuclear cell suspensions as prepared above, it was necessary to remove these red cells. This was done by hypotonic lysis.

Composition of lysis medium

Ammonium chloride 0.8% (w/v)	800 mg
Tri-sodium EDTA 0.1% (w/v)	100 mg
Potassium dihydrogen phosphate 0.01 (w/v)	10 mg
Sterile distilled water to 100 mls	

The cell pellet was incubated with 2 ml of the above medium for 5 minutes at 4°C, followed by the addition of 10 mls TC 199 followed by centrifugation. Two further washes in TC199 were carried out prior to resuspending the mononuclear cells in the appropriate medium for further experiments. Viability was always >95%.

1.3 Papainisation of effector cells

This was performed as for red cell papainisation (see below). Mononuclear cell suspensions obtained as above were centrifuged to a pellet containing approximately 5×10^6 mononuclear cells, and the cells resuspended in 1% papain in normal saline for 4 minutes at room temperature. The cells were then washed three times in TC 199, resuspended in TC 199/10% AB serum. Viability was always greater than 95%.

1.4 Pre-incubation of effector cells with anti-D

$5-10 \times 10^6$ mononuclear cells obtained as above were centrifuged to a pellet, and resuspended in 100 µl anti-D serum or IgG fraction of anti-D serum. After mixing, the cells were incubated at 37°C for 1 hour and then washed x 2 in TC 199 before being resuspended in TC 199/10% AB serum.

2.0 IDENTIFICATION OF MONOCYTES

Although the mature monocyte has recognisable morphological features (>10 microns diameter; reniform nucleus; abundant granular cytoplasm; irregular cytoplasmic outline with pseudopodia) it can be difficult, if not impossible to distinguish large lymphocytes (>10 microns diameter) from monocytes on the basis of morphology alone. Furthermore, it is likely that there will be some monocytes which cannot be distinguished from smaller lymphocytes on this basis. A number of techniques have therefore been used which detect monocytes on the basis of functional characteristics such as adherence, phagocytosis and histochemical staining.

2.1 Phase contrast microscopy

One drop of cell suspension (approx. 5 x 10^6/ml) was used to fill the chambers of a Neubauer haemocytometer and then examined under phase contrast light at the highest convenient magnification . The granular cytoplasm of neutrophils and monocytes shows up clearly in contrast to that of lymphocytes which have a much lighter and more uniform appearance. Since the nuclear morphology can also be seen, polymorphs are easily recognised. The percent monocytes were estimated from a count of at least 200 cells.

2.2 Toluidine Blue staining

The supervital stain Toluidine Blue is taken up by living cells so that cytoplasmic and nuclear morphology may be clearly distinguished.

Prepared slides were made by making a 0.5%

suspension of Toluidine Blue in absolute methanol, adding one drop to a clean dry microscope slide followed by drying at 37°C in an incubator. A thin deposit of Toluidine Blue remains so that one drop of cell suspension can be added directly to the prepared dried slides, gently mixed, a cover slip added, and the cell suspension examined after allowing 2-3 minutes for the cells to take up the dye. The percentage monocytes was assessed by counting at least 200 cells.

2.3 Latex particle phagocytosis

Monocytes can be identified following the ingestion of a suitable indicator material. Latex particles (1μ diameter; Dow-latex) are convenient in that they are inert, readily phagocytosed and can be easily identified within the cell under light microscopy.

Two to three drops of 1/100 dilution of latex suspension (as obtained from manufacturer) were added per ml TC 199/10% AB serum containing mononuclear cells at 5-10 x 10^6/ml. The mixture was incubated at 37°C for 30-60 minutes with frequent mixing. Excess latex particles were removed by washing in TC 199. The cells were examined under light microscopy, monocytes being identified by the presence of latex particles within the cytoplasm. Neutrophil polymorphs also ingest particles but they were distinguished from the monocytes on the basis of nuclear morphology. The percentage monocytes was calculated from a counting of at least 200 cells.

2.4 Neutral red phagocytosis

1% neutral red in normal saline was freshly prepared and an equal volume mixed with cell suspensions containing 1-2 x 10^6 mononuclear cells. After incubation at 37°C for 30 minutes the cells were washed and resuspended in TC 199 and examined under the microscope. Monocytes appeared dark red, having phagocytosed the dye from the medium. The percentage monocytes was assessed by counting the 200 cells.

2.5 Non-specific esterase stain

The histochemical assessment of non-specific esterase cytoplasmic activity is specific for the monocyte (Li et al 1973).

The substrate, α-napthyl butyrate is hydrolysed by monocytes and the diazonium salt, hexazotised pararosanalin, couples this to form an insoluble intracellular reddish brown deposit which can be readily identified on fixed cell smears (see fig. 2.5). The advantage of this technique is that it is objective, permanent slides are obtained and a batch of slides can be stained and counted after fixing some days earlier.

Stock Solutions

(1) One ampoule α-napthyl butyrate (0.25 ml) (Sigma) is made up to 48 ml in 1/4 Dioxan (Koch-Light).

(2) 1 g pararosanalin HCl (Sigma) in 30 mls 2 N HCl

(3) 1 g sodium nitrite ($NaNO_2$) (BDH Chemicals Ltd) in 30 ml H_2O (make up fresh each time).

(4) 0.4 M sodium cacodylate (BDH Chemicals Ltd).

(5) 10% Tween 20.

(6) 98% methanol.

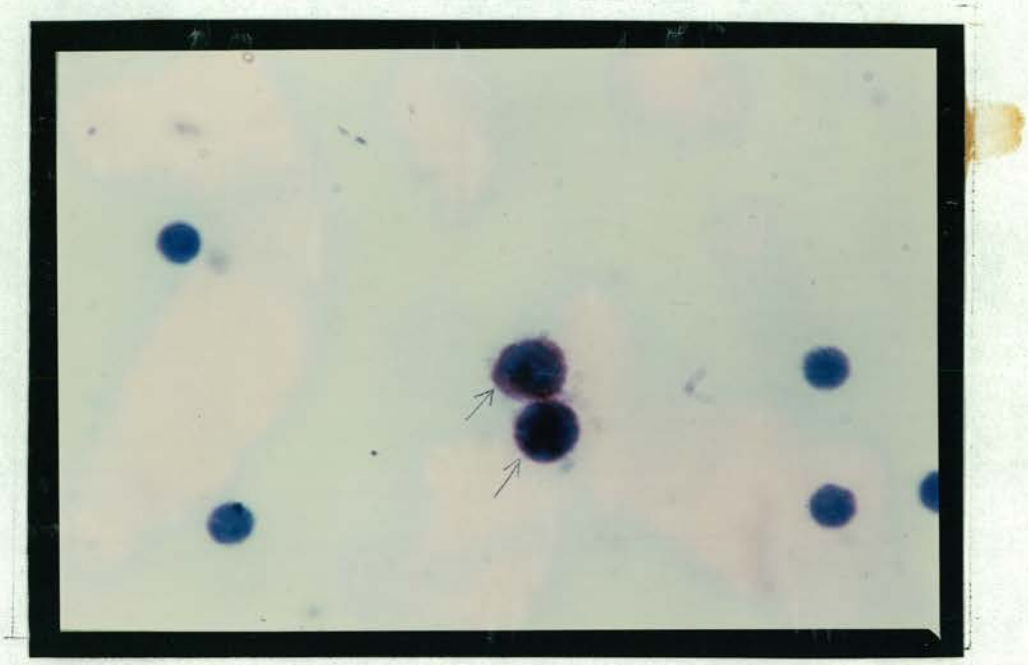

Fig. 2.5 Non-specific esterase stain with two positive monocytes shown (see also fig. 2.1.1 p. 226)
(x 200 magnification)

Solution A

Mix 0.8 ml of (2) with 0.8 ml of (3) in a 50 ml glass measuring cylinder and shake for 30 seconds (solution goes yellow-brown) add 4-5 mls of (4) and correct the pH to 6.0 with NaOH. Make up to 15 ml with distilled water and add 0.1 of (5).

Solution B

Add 2 mls of (1) and 3 mls of (6) to solution A and make up to 40 mls with distilled water.

Only solutions (1), (2), (4), (5) and (6) are stable. Solution (3), solutions A and B must be prepared immediately prior to staining.

Staining Technique

A drop of cell suspension was smeared on a microscope slide and the film allowed to dry before fixing in formalin vapour for 30 seconds. The slides were then washed in distilled water and dried and could be left at this stage for up to 5 days before proceeding to the esterase staining.

Slides were then stained with solution B, as prepared above, for 10-25 minutes. The actual time was found experimentally by examining the slides, since solution (1) (α-napthyl butyrate) deteriorates slowly on storage and the staining time needs to be prolonged accordingly. Slides were then washed in distilled water, counter-stained with Giemsa (10% in phosphate-buffered-saline (PBS) pH6.8) for 3-5 minutes, and then decolourised with PBS for 2 mins. dried in air and the slides preserved by spraying with liquid cover-glass (Trycolac, Aerosol

Marketing and Chemical Co. Ltd). The percentage monocytes were assessed by counting at least 200 cells. Slides prepared after depletion of monocytes contained very few esterase-positive cells and in these cases more than 1000 cells were scanned.

2.6 Anti-monocyte serum staining

This was kindly performed by Dr. A.E. Stuart according to the method published by Stuart et al, (1976).

In brief, smears of mononuclear cell suspensions were made on clean microscope slides, air dried, fixed in 90% ethanol and then stored at 4°C until ready for staining. The smears were washed with P.B.S. for 15 minutes followed by incubation with 1/100 dilution of rabbit anti-human monocyte serum (AMS) or with normal rabbit serum (control) for 30 minutes at 37°C in a moist slide box. The preparations were then rinsed and washed in PBS x 3 for 15 minutes each time and then incubated at 37°C for 30 minutes with 1/20 dilution of FITC-conjugated goat anti-rabbit IgG. After a further three washes in P.B.S., the preparations were sealed under a coverslip in one drop of P.B.S./glycerol (1:9) and examined under incident-light fluorescence microscopy using a Leitz Ortholux microscope with an HBO 200 mercury vapour lamp, BG38 and BG12 excitation filters and a K510 suppression filter.

3.0 REMOVAL OF MONOCYTES FROM CELL SUSPENSIONS

3.1 Removal by plastic culture dishes

Mononuclear cell suspensions prepared as above (para. 1.0) were washed in TC 199 and resuspended in TC 199/20% AB serum at a concentration of not more than 5 x 10^6/ml, added to a Falcon plastic flat-sided tissue culture flask (no. 3012) and the total volume made up to 20 ml by the addition of TC 199/20% AB serum. The flasks were incubated flat at 37°C for 90 minutes, then turned over on the other side for a further 90 minutes giving a total incubation time of 3 hours. The flasks were gently shaken to resuspend non-adherent cells which were recovered by centrifugation. After a further two washes in TC 199, the cells were counted and adjusted to the appropriate number in TC 199/10% serum. The cells were always >95% viable and usually >99% viable. The degree of 'contamination' by monocytes is discussed in chapt. IV - results, but was less than 5%.

3.2a Removal by nylon wool columns

Sterile nylon wool (Leuko-pak, Fenwal Laboratories, Ltd.) was washed with TC 199/10% AB serum to remove trapped air bubbles, and the equivalent of approx. 500 mg dry fibre packed into a sterile disposable 10 ml syringe (Plasti-pak), fitted with a disposable 3 way tap (Pharmaseal). The column was flushed with 10-20 ml TC 199, the top sealed with parafilm (American Can Company) and allowed to equilibrate at 37°C for 1 hour. The column was then washed with 30 mls pre-warmed TC 199,

the mononuclear cell suspension added in 2-3 mls of TC 199/10% serum, and the columns incubated horizontally at 37°C for 30 mins. The columns were rotated through 180° and incubated for a further 30 minutes. The non-adherent cells were eluted in the vertical position with pre-warmed TC 199. 40 mls TC 199 was used initially, but 20 ml was found to be sufficient to recover more than 90% of the non-adherent cells. The cells were recovered by centrifugation, washed twice in TC 199 and finally resuspended at the desired concentration in TC 199/10% serum. Viability of the cell suspensions was between 95-99%. The average recovery of cells and the effects on various lymphocyte subpopulations are described in chapt. IV - results.

3.2b Recovery of adherent cell population

The nylon wool was recovered from the columns into a beaker containing 10-20 mls warm medium 199/10% AB serum and gently teased with glass rods. The supernatant suspension containing recovered cells was collected into sterile plastic tubes (Nunc) and the process repeated twice. The cells were recovered by centrifugation, pooled, washed in TC 199, counted and finally resuspended in TC 199/10% serum.

The viability of the recovered cell suspension was usually poor (approx. 70%) with much cellular debris and ruptured cells present. For functional assays, the cell suspensions were adjusted to give the required numbers of viable cells per culture.

Recovery of cells from the columns was poor (approx. 10%) presumably due to the fact that cells remained

adherent to the nylon or were irreversibly damaged after removal so that they were non-viable. Slightly better recovery was achieved using calcium and magnesium-free complement-fixation-buffer instead of TC 199 - possible because these ions are necessary for phagocytosis and adherence and this medium was used in place of TC 199 in later experiments.

3.3 Removal of monocytes by Carbonyl Iron

Phagocytic cells can be removed from cultures by allowing them to ingest Carbonyl Iron particles followed by removal of these cells using a magnet.

Mononuclear cell suspensions obtained as in para. 1.0 were adjusted to approximately 5 x 10^6/ml in TC 199/10% AB serum in a Nunc. plastic tube and to this is added approximately 10 mg of Iron ex Carbonyl Iron (Koch-Light Laboratories). The suspension was then incubated at 37°C for 40 minutes with frequent mixing. After incubation the cells were thoroughly mixed and Iron-containing cells "pulled" to the bottom of the tube with a large hand magnet. The supernatant cell suspension was removed, washed x 1 in TC 199 and adjusted to the appropriate concentration in TC 199/10% AB serum. Viability was 95-99%.

4.0 IDENTIFICATION OF T LYMPHOCYTES

T lymphocytes have surface receptors for sheep red cells and will therefore bind them to form an erythrocyte rosette. Normally, the binding of sheep RBC (E) to T lymphocytes is relatively weak and the treatment of E with aminoethyliso-thiouronium-hydrobromide (AET) stabilises the binding giving a more reproducible test with maximum identification of T cells as E-AET rosettes. The method used was modified from that of Kapland & Clark, 1974. Two mls of sheep RBC stored in 50% Alsever's solution (Wellcome) were washed x 3 in 10 mls of saline and hard-packed by centrifugation. To 1 volume of washed packed sheep RBC was added 4 volumes of AET solution (0.402 g of AET in 10 mls distilled water, adjusted to pH 9 with 4N NaOH). The mixture was shaken and incubated at 37°C for 15 minutes with repeated mixing. The cells were then washed 3 times in normal saline and once in TC 199 and finally resuspended in TC 199/20% foetal calf serum (FCS) (Wellcome) to give a 10% suspension of E-AET. These cells could be stored at 4°C for up to one week prior to rosetting tests.

Mononuclear cell suspensions were prepared as in para. 1.0 and phagocytic cells were identified by latex particles ingestion as described in para. 2.3 The cell suspension was finally washed and adjusted to 2 x 10^6/ml in TC 199.

The rosetting procedure was performed by adding 0.5 mls of mononuclear cell suspension to 0.25 ml of FCS followed by 2-3 drops of E-AET. The mixture was

thoroughly shaken, centrifuged gently at 25 g for 5 minutes and then incubated at 4°C. Maximum rosette formation was observed within 1 hour of incubation, but for convenience most incubations were carried out overnight (18 hrs). The cell pellet was gently resuspended using a wide bore Pasteur pipette. An estimation of the percentage rosette-forming cells (3 or more RBC bound to the mononuclear cell surface) was made immediately on cell suspensions by adding 1 drop to a Toluidine Blue microscope slide, and examining as in section 2.2. Alternatively, permanent slides could be prepared by making smears, drying in air, fixing in absolute methanol for 2 minutes, followed by staining with May-Grunwald-Giemsa. When dry, the slides were sprayed with liquid cover-glass (Trycolac) and these permanent smears could then be examined at leisure. All non-rosetting lymphocytes were identified and counted and contaminated cells ie. monocytes and polymorphs, were excluded on morphological grounds and on the basis of latex particle ingestion.

4.1 T lymphocyte depletion by E-AET rosette sedimentation

Mononuclear cell suspensions prepared as in para. 1.0 were adjusted to 10 x 10^6 /ml in TC 199. The cells were mixed with equal volume of 1% E-AET and cell-to-cell contact enhanced by centrifugation at 25 g for 5 minutes. The cell pellet was then incubated at room temperature for 30 minutes, gently resuspended with a wide bore pasteur pipette, recentrifuged and incubated at 4°C for 2 hours. The pellet was then gently resuspended and an aliquot taken to estimate the numbers of E-AET

rosette-forming cells and the remainder layered over Ficoll-Triosil (specific gravity 1.077) and centrifuged at 1000 g for 15 minutes. The cells at the interface and pellet were then collected separately and aliquots taken for estimation of rosette forming cells. The remainder of the cells were then washed x 1 in TC 199 and the RBC removed by hypotonic lysis as described in section 1.2. The unseparated aliquots were treated identically. The cells were then washed x 3 in TC 199, checked for viability (95-99%) and adjusted to the appropriate cell number in TC 199/10% AB serum.

5.0 IDENTIFICATION OF B LYMPHOCYTES

5.1 Surface membrane immunoglobulin (SIg) bearing cells

B lymphocytes can be identified by the demonstration of surface membrane immunoglobulin which can be shown by an indirect technique using an anti-human immunoglobulin serum raised in a particular animal species followed by a fluorescent-labelled anti-species serum (Seligmann, Preud'homme & Grouet 1973).

Approximately 5 x 10^6 latex-treated mononuclear cell suspension were prepared as above (1.0) and washed with ice-cold phosphate buffered saline (PBS) pH 7.3 containing 0.2% sodium azide. The supernatant following washing was removed as thoroughly as possible and the cells resuspended in 50 µl of 1/10 goat anti-human immunoglobulin (Behringwerke). The cells were incubated at 4°C for 30 minutes and then washed 3 times with 10 mls cold PBS, and the supernatant thoroughly removed from the pellet. The cells were resuspended in 50 µl of 1/10 FITC-rabbit anti-goat immunoglobulin serum and incubated for 30 minutes at 4°C. The cells were washed 3 times in 10 mls cold PBS, the supernatant removed and the cells fixed by the addition of 200 µl of 1% paraformaldehyde for 4 minutes at room temperature. The cells were washed twice in PBS and then mounted in PBS/glycerol and the edges of the coverslips sealed with clear nail varnish to prevent drying. Slides were examined immediately or could be stored in the dark at 4°C for several days before being examined under incident light fluorescence microscopy. By examining the fields alternately under phase contrast and incident light fluorescence (as in para. 2.6) the total number of lymphocytes can be counted followed by an estimate of the number of

fluorescing B lymphocytes (excluding cells containing latex particles). The percent B lymphocytes was estimated by counting at least 200 lymphocytes.

5.2 C3 receptor bearing cells

B lymphocytes have membrane receptors for the complement component C3 which can be detected by rosette formation with complement-coated red cells. The indicator cells are sheep RBC (E) treated with IgM anti-RBC antibody (A) and human complement (C) (EAC3). Since polymorphs and monocytes have a C3 receptor, they will also form EAC3-rosettes and are therefore distinguished from B lymphocytes by latex particle phagocytosis. The method used was adapted from Bianco et al (1970).

0.5 ml SRBC stored in Alsever's solution (50% suspension) (Wellcome) was washed and resuspended in 5 ml normal saline. 30 µl of rabbit IgM anti-SRBC serum (Flow Laboratories) was added and the cells incubated at 37°C for 20 minutes with occasional mixing. The anti-SRBC serum must be diluted such that direct agglutination of SRBC does not occur. The cells were then washed 3 times and resuspended in 10 mls of saline. 0.3 ml of fresh human group AB serum (as a source of complement) was then added and the cells incubated for a further 20 minutes at 37°C with frequent mixing. The cells were then washed 3 times and resuspended in 5 mls of saline giving a 10% suspension. Fresh EAC3 suspensions were prepared on the day of testing. Mononuclear cell suspensions were prepared (as in para 1.0) and phagocytic neutrophils and monocytes identified by the addition of latex particles (para. 2.3). The cells were adjusted to approximately 2 x 10^6/ml and 2-3 drops of EAC3 suspension

added to 0.5 ml mononuclear cell suspension, mixed and centrifuged at 25 g for 5 minutes and incubated at 37°C for 40 minutes. The cells were resuspended using a Pasteur pipette and slides prepared for examination as described in 4.1. In order to facilitate identification of the central cell of the rosette, Toluidine Blue treated slides as described in para 2.2 were used. The % EAC3 rosetting cells (≥ 3 RBC/cell) was estimated by counting at least 200 cells, exclusing monocytes and polymorphs.

5.3 Depletion of C3 receptor-bearing cells by EAC3 rosette sedimentation

Mononuclear cell suspensions were prepared following nylon wool passage as described above and an aliquot set aside. The remaining mononuclear cells were diluted to 2×10^6/ml and 2 drops of 10% EAC3 added for every 0.5 ml mononuclear cell suspension. After mixing, the cells were centrifuged into contact at 25 g for 5 minutes and then incubated at 37°C for 40 minutes. The cell mixture was then resuspended and an aliquot taken for estimation of EAC-rosettes. The remainder was layered over Ficoll-Triosil (specific gravity 1.077) and centrifuged at 1000 g for 15 minutes. Cells at the interface and the cell pellet were then harvested separately to give EAC3-depleted and EAC3-enriched populations respectively. Aliquots were taken for estimation of the EAC3-rosette forming cells from each population. The cells from the pellet, the interface, and the original unseparated aliquot were then suspended in hypotonic lysis medium (2 mls at 4°C for 5 minutes) and then washed x 3 in 10 mls TC 199. The cells were then checked for viability (95-99%) and adjusted to

the appropriate concentration of TC 199/10% AB serum for K-cell assays (see below).

5.4 Depletion of C3 receptor bearing cells by EAC3 monolayers

EAC3 monolayers were prepared as in para. 7.2 and mononuclear cell suspensions depleted of monocytes as in para. 3.2a. Six million mononuclear cells were added to each monolayer which was then centrifuged at 200 g for 5 minutes, and incubated at 37°C for 1 hour. The non-adherent cells were recovered as described in para. 7.2. Aliquots of each population were assessed for EAC3 rosettes and K-cell haemolysis (see below).

In some experiments, a second absorption as above was carried out on fresh monolayers.

5.5 Fc-receptor-bearing cells

A subset of B lymphocytes and monocytes, polymorphs and K-cells possess a receptor for the Fc portion of IgG. These cells can be identified by rosette formation with indicator RBC (E) coated with IgG (A) giving EA-rosettes.

0.5 ml sheep RBC were washed and resuspended in 5 ml normal saline. 50 µl IgG anti-SRBC (Flow Laboratories) was added (sub-agglutinating dose) and the cells incubated at 37°C for 20 minutes. The cells were then washed 3 times and resuspended in 5 ml normal saline giving a 10% (v/v) suspension. Fresh suspensions were made up on the day of testing. Mononuclear cells were prepared as in para. 1.0 with latex particles added as in para.

2.3 and adjusted to 2 x 10^6/ml.

2-3 drops of 10% EA were added to 0.5 ml mononuclear cell suspension, followed by centrifugation at 25 g for 5 minutes and incubation at 37°C for 40 minutes. The cells were resuspended and examined under the microscope on Toluidine Blue slides (para. 2.2). The percentage EA rosetting cells (> 3 RBC/cell) was estimated by counting at least 200 cells, excluding monocytes and polymorphs.

5.6 Depletion of Fc-receptor-bearing cells by EA monolayers

Monolayers were prepared as in para. 7.1 using human D positive RBC sensitised with anti-D. Six million mononuclear cells (para. 1.0) were added to each monolayer, followed by centrifugation at 200 g for 5 min and incubation at 37°C for 1 hour. The non-adherent and adherent cells were recovered as described in 7.2. Aliquots of each population were assessed for EA rosettes and K-cell haemolysis (see below).

In some experiments a second absorption as above was carried out on fresh monolayers.

6.0 PREPARATION OF HUMAN RED CELLS

Venous blood samples were obtained from normal donors or laboratory personnel of known ABO and rhesus groups. The rhesus types were confirmed by routine blood banking methods and the following types were most commonly used; the Wiener notation is used for convenience in the following sections.

group O rhesus negative	O rr	(cde/cde)
group O rhesus positive (homozygous for D)	O R_1R_1	(CDe/CDe)
	O R_1R_2	(CDe/cDE)
	O R_2R_2	(cDE/cDE)
group O rhesus positive (heterozygous for D)	O R_1r	(CDe/cde)

In early experiments, blood was collected into acid-citrate-dextrose (ACD, NIH-A formula) 1 vol. to 9 vol. blood, and then stored at $4^{o}C$ until required. Blood stored in this way was suitable for up to one week as target cells for the haemolytic assay and up to two weeks for other purposes eg. anti-D titration, absorption studies. Better storage was obtained with a medium used at the Central Laboratory of the Netherlands Red Cross, Amsterdam (Dr. Erna van Loghen, personal communication - see below). Blood collected as above (1 vol. storage medium to 9 vol. blood) maintained the viability of target RBC for the lytic assay for up to 3 weeks, and up to 5 weeks for other purposes. Wherever possible however, fresh red cell targets were obtained each week since the spontaneous ^{51}Cr release increased with storage

time (see below). Group O RBC were always used, and for most experiments O R_1R_1 were used as targets for the K-cell assay.

6.1 "Amsterdam storage medium"

0.02 M Na_2 EDTA	0.744 g
0.02 M Na_2 HPO_4 $2H_2O$	0.356 g
Bovine albumin	1 g = 3.3 ml 30% BSA
Chloramphenicol	100 mg
glucose	1 g
distilled water to 100 ml	

9 vols. whole blodd are added to 1 vol. storage medium, mixed by inversion and stored at 4°C until required.

6.2 Papainisation

5 drops of RBC suspension as stored above were mixed with an equal volume of 1% papain in sterile saline (BDH papain, papaya) for 4 minutes at room temperature. The RBC were then washed 4 times in 10 mls TC 199, resuspended and counted.

The duration of papainisation and the actual concentration of papain was found not to be critical. Equally good results were obtained with Löw's cystein-activated papain method which contains approximately 0.5% papain and cystein HCl in Sorensen's buffer (pH 5.4), or with 1% bromelin. The time of papainisation was also not critical in that between 2-10 minutes was satisfactory. With longer times, problems were seen with non-specific agglutination in titration assays and the procedure was therefore standardised as above.

It was found from preliminary experiments that papainisation was equally satisfactory if performed before or after ^{51}Cr labelling. To avoid unnecessary handling of radio-active material, papainisation was carried out prior to ^{51}Cr labelling (see below).

6.3 Labelling of RBC with ^{51}Cr

20×10^6 papainised or untreated RBC in 100 µl TC 199/10% AB serum were incubated at 37°C for 1 hour with 200 µCi ^{51}Cr ($Na_2\ ^{51}CrO_4$ - CJS4, Amersham) adjusted to 50 µCi per 10 µl with sterile saline). After incubation the RBC were resuspended, washed x 4 in 10 mls TC 199/ 10% AB serum and adjusted to the appropriate concentration for K-cell assays (see below).

Target cells pre-sensitised with anti-D were prepared by incubating 20×10^6 papainised or untreated RBC in 100 µl of the anti-D containing material (either heat-inactivated serum or IgG fraction thereof) and incubated with 200 µ ^{51}Cr as above. The sensitised labelled RBC were then washed and processed as above. Papainised RBC agglutinate after sensitisation with anti-D and were therefore vigorously resuspended prior to counting and dispensing in order to break up the agglutinates.

Extensive preliminary experiments were performed to determine the ideal conditions for maximum ^{51}Cr uptake by RBC with minimum spontaneous ^{51}Cr release during culture conditions. Although RBC stored and labelled in ACD gave much better ^{51}Cr uptake, there was a much more variable spontaneous ^{51}Cr release during the

incubation time, from 5-40% of the original intracellular ^{51}Cr. The spontaneous release was of course corrected during the calculations of cytotoxic activity (see below) but a high spontaneous ^{51}Cr release decreases the sensitivity of the assay. The variable results with ACD stored and labelled RBC was probably due to osmotic and pH changes undergone by the red cells following the change from ACD to TC 199 during the washing process, and this was minimised by labelling and washing in TC 199 throughout.

The spontaneous ^{51}Cr release with labelled RBC was less than 5% with freshly drawn and processed cells but was found to gradually increase with storage time of the red cells in vitro. Cells stored in Amsterdam storage medium were therefore changed at weekly intervals wherever possible.

7.0 PREPARATION OF RBC MONOLAYERS BY POLY-L-LYSINE

Red cell monoalyers were prepared on Falcon plastic petri dishes (35 mm x 10 mm) by the poly-l-lysine method adapted from that of Dennedy and Axelrad (1971).

2 ml poly-l-lysine (Sigma, M.W. 30,000) at 50 µg/ml was added to each petri dish and incubated at room temperature for 1 hour. Poly-l-lysine was freshly diluted in PBS pH 7.3 from a stock solution of 1 mg/ml kept at 4°C. The petri dishes were then washed 3 times with PBS, 2 mls 1% appropriate RBC suspension added (see below) and incubated for 1 hour at room temperature, Non-adherent RBC were then washed off with PBS and the monolayers checked microscopically for even distribution of adherent cells.

Sheep EA and EAC3, plain, papainised and AET treated human RBC formed even monolayers by the above method. The RBC were prepared as described above and adjusted to 1% (v/v) suspensions in PBS. Better results were obtained with fresh RBC, but storage at 4°C up to one week gave satisfactory results.

7.1 Anti-D sensitised monolayers

Monolayers were prepared as above with papainised RBC (para. 6.2). The monolayers were then incubated with 100 µl Louden anti-D serum in 2 ml PBS for 1 hour at 37°C, followed by 3 washes with PBS and were then ready for use.

7.2 Depletion of cell populations by monolayers

Monolayers were prepared as above with the appropriate RBC suspensions. Mononuclear cells were prepared with adherent monocytes removed (para. 3.2a) and adjusted to 3 x 10^6/ml in TC 199/10% AB serum. Aliquots were taken to assess the numbers of receptor bearing cells. 2 ml suspension was then added to each monolayer which was centrifuged at 200 g for 5 minutes to bring the mononuclear cells and RBC into contact, followed by incubation for the appropriate time and temperature for each type of monolayer (see appropriate section). Non-adherent cells were recovered by gentle agitation of the plates and recovery of the supernatant This was repeated and suspensions pooled, washed, counted and adjusted to the appropriate number in TC 199/10% AB serum. In some experiments the adherence to monolayers was repeated as above, and the cells recovered after two adherence incubations. The adherent population was recovered by hypotonic lysis (para. 1.3) and gentle scraping with silicone rubber. The cells were then washed and processed as above.

Aliquots of the original cell suspensions, the enriched and depleted populations were assessed for appropriate receptor-bearing populations to estimate the efficiency of depletion and aliquots were prepared for K-cell assays as described below.

8.0 PREPARATION OF ANTISERA

8.1 Anti-D sera

Several plasmapheresis packs were obtained from a donor (Mrs. S. Louden) who aborted due to haemolytic disease of the newborn as a result of anti-D immunisation. Despite the relatively low amounts of anti-D in this material as determined by routine methods (see below) the biological potency of this antibody had been "proven". This material was therefore used extensively either as a serum or as a starting material for the preparation of an IgG fraction containing anti-D (see below).

Other anti-D containing sera were obtained from routine samples received in the Blood Transfusion Centre for ante-natal screening during pregnancy or from male and female volunteers who were under going plasmapheresis for the production of anti-D for therapeutic use.

Plasma was converted to serum by the addition of one drop of thrombin solution (Parke-Davi s) per ml of plasma. After incubation at 37°C for one hour, the material was centrifuged and the clear supernatant transfered into clean sterile containers. The serum was then heat-inactivated at 56°C for 30 minutes to destroy Complement activity. The specificity of the anti-D was confirmed by testing against a panel of standardised red cells by conventional saline, enzyme and indirect antiglobulin methods and, where possible, by the automated autoanalyzer quantitation method (Gunson et al 1972) using the National Institute of Biological

Standards and Controls standard anti-D for calibration.

8.1a "Louden" anti-D serum

This serum obtained as above contained only anti-D, with no other red cell antibodies being detected by any of the above methods. Quantitation of anti-D revealed the following results:

Saline 37°C titre	-	nil
Papain enzyme titre	-	1/16
Indirect antiglobulin titre	-	1/32
Autoanalyzer quantitation	-	4.16 µg/ml

IgG sub-class determinations kindly performed by Dr. C.P. Engelfriet (Central Laboratory of the Netherlands Red Cross, Amsterdam) revealed the following results:

Anti-IgG_1 titre	-	1/512
Anti-IgG_2 titre	-	1/32
Anti-IgG_3 titre	-	nil
Anti-IgG_4 titre	-	nil
Anti-IgA titre	-	nil
Anti-IgM titre	-	nil

This patient's serum therefore contains anti-D with only IgG antibodies mainly of the IgG_1 sub-class.

This material, and the IgG fraction prepared from it, was used for the majority of experiments performed on the *in vitro* investigations on the K-cell mechanism by anti-D.

8.1b IgG fraction of Louden anti-D

This was prepared by the method of Webb 1972 with slight modifications and was kindly done by Dr. D.S. Pepper (Edinburgh Blood Transfusion Service). Plasmapheresis packs containing 200-250 mls of material as above, were used as a starting material.

DEAE-Sephadex-A50 (Pharmacia) was allowed to swell in 0.1 M phosphate pH 6.5 over 3 hours with constant stirring and the pH maintained at 6.5 by the addition of NaOH or HCl, as required. The swollen gel was then washed on a Buchner filter and resuspended in 0.01 M phosphate pH 6.5 and the pH adjusted as necessary to 6.5.

For IgG production, 5 volumes of pre-swollen gel were mixed with 1 vol. of plasma, mixed for 30 minutes, the gel compressed by centrifugation, and the clear supernatant containing the IgG recovered and freeze dried.

Two separate lots were prepared, the first being designated IgG I (see table 8.1b for details) and the second being designated IgG II or IgG III depending on the protein concentration at which the material was dissolved (see table 8.1b).

The dried powder was reconstituted in TC 199 to give a nominal protein concentration as noted in table 8.1b. This material was clarified by centrifugation at 3000 g for 20 minutes and then dialyzed against TC 199 at 4°C overnight. The recovered material was adjusted to the starting volume, centrifuged at 3000 g for 15 minutes to clarify and then filtered through a bacterial filter (0.22 μ cut-off point; Millex, Millipore

Co. Ltd.) and stored in aliquots at -40°C until ready for use.

The details on the three batches of material used are as follows:

Table 8.1b

Designation in text	Protein* concentration	Anti-D quantitation** Saline titre	Enzyme titre	IAGT titre	Auto-analyzer (μg/ml)
IgG I	not tested	nil	1/1800	1/2048	approx. 240
IgG II	10 mg/ml	nil	1/8	1/32	1.10
IgG III	20 mg/ml	nil	1/8	1/64	2.24

* amount of freeze-dried material reconstituted per ml of medium

** quantitation against O R_1R_1 RBC

8.2 Dilution of anti-D for culture

Anti-D serum or its IgG fraction was usually diluted in medium 199 supplemented with AB serum and antibodies to give the required final concentration. 50 μl of anti-D containing material was added at 3 times the desired concentration to K-cell cultures with a final culture volume of 150 μl.

For experiments where anti-D sera were adjusted to the same equivalent anti-D concentration in μg/ml, the diluent was antibody-free AB serum so that the final total immunoglobulin concentrations were comparable for each dilution of the anti-sera.

8.3 Absorption of anti-sera (anti-D)

Group O rhesus negative (rr) and O rhesus positive (R_1R_1) red cells were washed in normal saline and hard-packed by centrifugation. 1 vol. of anti-D containing material (1-2 mls) was added to 1 volume of packed RBC of each type, incubated at 37°C for 40 minutes, centrifuged and the supernatant removed. After an aliquot had been taken for functional studies and anti-D titres, the bulk of the supernatant was again incubated with an equal volume of packed RBC as above, and the supernatant recovered. The anti-D titre was assessed before and after each absorption and the absorbed and unabsorbed fractions were tested for activity in K-cell cultures against O R_1R_1 target RBC (see below).

9.0 CULTURE CONDITIONS FOR K-CELL HAEMOLYTIC ASSAY

Experiments were always set up in triplicate cultures for each variable under examination and including the following controls -

1) ^{51}Cr labelled RBC in TC 199/10% serum to assess spontaneous ^{51}Cr release.

2) ^{51}Cr labelled RBC in the presence of anti-sera (or inhibitor or any other agent being tested), to assess potential toxic effects on the red cells.

3) ^{51}Cr labelled RBC in distilled water or 1% Triton X to assess maximum ^{51}Cr release possible.

4) ^{51}Cr labelled RBC without sensitising antibody in the presence of the putative effector cell population to assess any direct cytotoxicity.

The test cultures contained a known number of ^{51}Cr-labelled RBC targets (usually group O R_1R_1) a known number of mononuclear effector cells (with the effector/target ratio usually 10:1), and anti-D sensitising antibodies. The anti-D was either added directly to the cultures at the beginning of the incubation period as a known amount of serum or IgG fraction, or used to presensitise the RBC targets as described in section 6.3. The overall design of the assay procedure is shown in fig. 9.0.

The culture condition, unless otherwise stated in the text, involved incubation of the cell mixture at 37°C in a humidified atmosphere containing 5% CO_2/air for 18 hours (overnight).

During the evolution of this assay certain changes were made which did not influence the final result (see

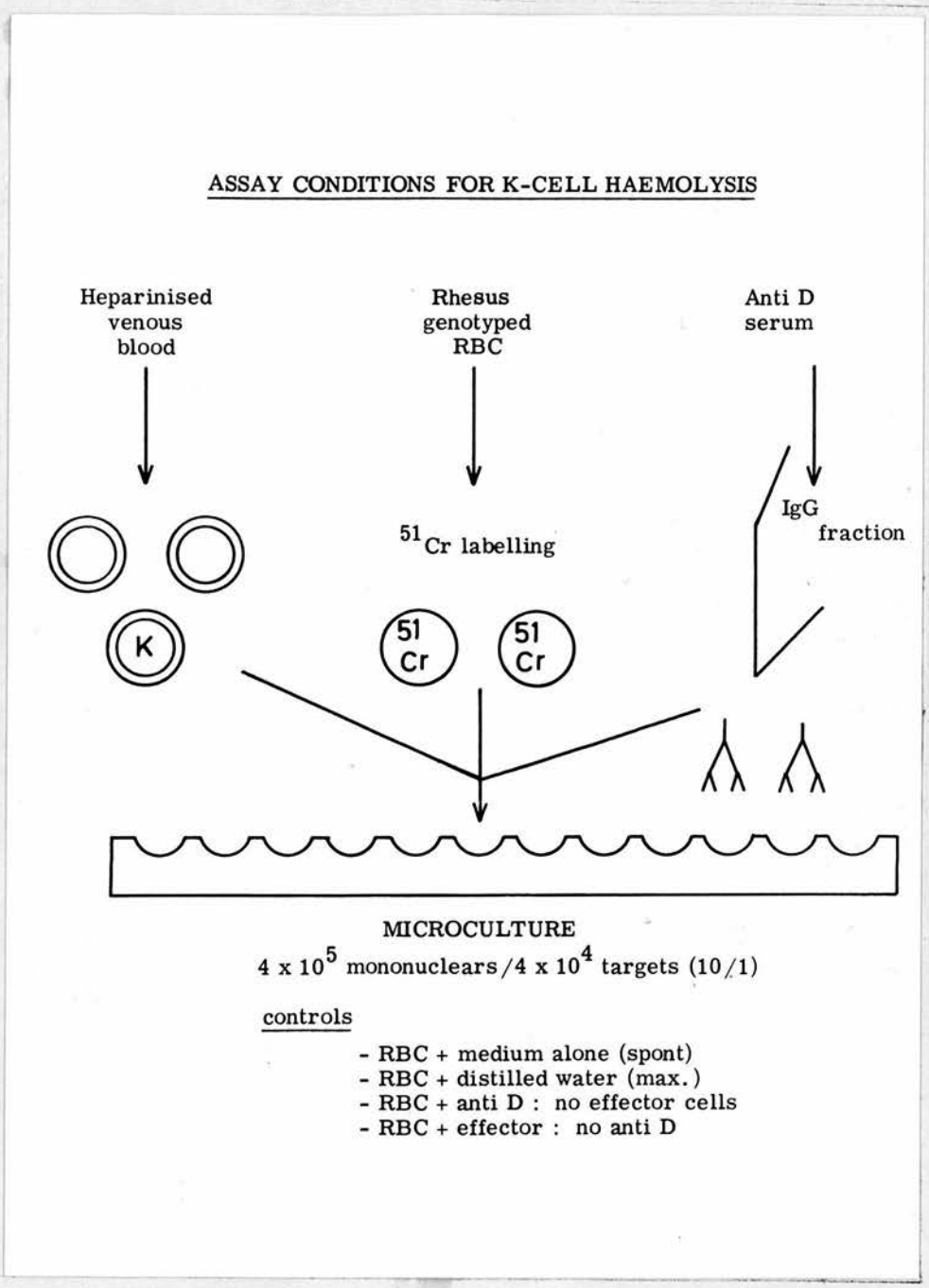

Fig. 9.0 Diagrammatic representation of K-cell assay

results section) but which involved slightly different methodology. These are described separately under the headings of 1) Macrotube assay 2) Microtube assay and 3) Microplate assay.

9.1 Macrotube assay

Mononuclear cell suspensions were prepared as above and adjusted to 2.5 x 10^6/ml in TC 199/10% AB serum. 400 µl aliquots (1 x 10^6) were dispensed using a micropipette (Oxford) in triplicate (with appropriate controls as above), into plastic centrifuge tubes (Luckhams LP3). Anti-D presensitised ^{51}Cr labelled RBC were prepared as above (section 6.4) adjusted to 1 x 10^6/ml in TC 199/10% AB serum and 100 µl dispensed per culture (1 x 10^5). The effector-to-target (E/T) cell ratio was therefore 10:1 and the final culture volume was 500 µl. In certain experiments, 100 µl anti-D serum was added directly to culture, in which case the effector cells (1 x 10^6) were added in only 300 µl of culture medium so that the final volume remained at 500 µl. In certain experiments where the E/T ratio was altered, the effector cell concentration was adjusted by appropriate dilution or concentration in culture medium so that the appropriate number of effector cells were added in 400 or 300 µl to give a final culture volume of 500 µl.

The spontaneous ^{51}Cr release and maximum ^{51}Cr release controls consisted of 100 µl of ^{51}Cr labelled RBC in 400 µl of medium alone (spontaneous release), 400 µl distilled water (maximum release) or 100 µl anti-D containing material and 300 µl TC 199/10% AB

serum (toxicity control). The cultures were then incubated as described above. At the end of the incubation period, the tubes were centrifuged at 400 g for 5 minutes and 200 µl aliquots of cell-free supernatant transferred to fresh LP3 tubes for the assessment of ^{51}Cr radioactivity(see below).

9.2 Microtube assay

This was performed under identical conditions as above except that the concentration of cells was reduced ten-fold so that 400 µl of effector cell suspension contained 1 x 10^5 cells, and 100 µl target red cell suspensions contained 1 x 10^4 cells. The cells were incubated with controls as above and processed identically.

This micro-method was extremely useful for defining variables such as the range of antibody dilutions or changes of E/T ratio where large numbers of replicate cultures were required from the same individual. Since relatively few effector cells were required these experiments could be carried out with the mononuclear cells obtained from 20-30 mls blood.

The only problem encountered was that of higher ^{51}Cr release from the red cells than with the macroculture and this is possibly related to some unknown protective effect of the larger cell numbers in culture.

9.3 Microplate assay

This was the final stage of development of the K-cell assay where triplicate cultures were conveniently set up on Cookes micro-titre round-bottomed trays containing 96 wells which could be processed and centrifuged as a

single unit and thus did not require the laborious process of labelling large numbers of culture tubes with the inherent problems of mislabelling, spillage and centrifuge time.

Effector and target cell suspensions were prepared as above, except that the final concentration of effector cells was 8×10^6/ml and that of RBC targets, 8×10^5/ml. 50 µl of effector cell suspension was dispensed using a micropipette giving an E/T ratio of 10:1 with 4×10^5 effector cells and 4×10^4 RBC per culture. If anti-D presensitised target cells had been prepared, the culture volume was made up to 150 µl by the addition of 50 µl of TC 199/10% AB serum. If anti-D was to be added directly to culture, then the appropriate dilution of serum or IgG fraction was added in 50 µl aliquots to the appropriate cultures to give a final volume of 150 µl. In some experiments, where inhibitors or other agents were being tested, these were added as either 50 µl or 100 µl to the final cultures giving a volume of 150 µl or 200 µl. The actual volume was chosen on the basis of convenience of preparation of the various concentrations of inhibitors. 200 µl was the maximum volume that could be conveniently accommodated in the culture wells.

The lower limit of 4×10^4 red cells was chosen as the minimum number which gave satisfactory total counts of ^{51}Cr with minimum spontaneous ^{51}Cr release. With less than 4×10^4 red cells, the spontaneous ^{51}Cr release tended to be high (in the region of 30%) which was higher than that seen with the microtube assay (10-20%) and possibly reflects the difference in culture volume (200 µl vs. 500 µl).

The spontaneous ^{51}Cr release and maximum ^{51}Cr release consisted of 4 x 10^4 RBC in 150 µl or 200 µl (as appropriate) of TC 199/10% AB serum or distilled water. The microplates were covered with a plastic lid (Cookes) wrapped in clear kitchen-foil to minimise evaporation and incubated in a humid 5% CO_2/air incubator for up to 18 hours overnight.

After incubation the plates were centrifuged for 5 minutes at 400 g in microplate tray holders (M.S.E.) and 100 µl of supernatant removed by micropipette into LP3 tubes. The radio-activity was assessed as below.

Centrifugation contact

In certain experiments, RBC targets and effectors were brought into contact at the beginning of incubation by centrifuging the microplates at 400 g for 10 minutes, followed by incubation at 37°C as above.

9.4 Alteration of incubation time

K-cell assays were set up as described above with a series of replicates. At a given time, one set of replicates were removed from culture and processed as above. The % spontaneous ^{51}Cr release and the % maximum ^{51}Cr release at this time was estimated from samples processed simultaneously with the K-cell assay cultures and the % specific lysis (see below) expressed in terms of these values. This was necessary since the % ^{51}Cr spontaneous release gradually increased with culture time and the maximum ^{51}Cr release was lower at the earlier culture times due to the fact that damaged RBC had not completely lysed and released all the ^{51}Cr.

9.5 Assessment of cytotoxicity

The principle of the cytotoxic assay is that ^{51}Cr is incorporated into the red cell and bound irreversibly to intracellular proteins (Bunting et al 1963). Furthermore, the ^{51}Chromate ion is converted to ^{51}Chromic ions to which the cell membrane is then impermeable. Extracellular release of ^{51}Cr therefore represents a breach in the red cell membrane and this has been taken to represent cell lysis. Preliminary experiments showed that % RBC lysis correlated with % ^{51}Cr release under the culture conditions used. The various controls as described above were used to estimate the spontaneous ^{51}Cr release and maximum releasable ^{51}Cr. This was compared with the ^{51}Cr release from RBC in the presence of effector mononuclear cells with or without antibody (see below).

The % ^{51}Cr release was calculated as follows: aliquots of cell free supernatant collected as above were assessed for radio-activity due to the presence of ^{51}Cr using a gamma-counter (Tracerlab-Gamma Guard 150) adjusted to maximum sensitivity for this isotope. The total supernatant ^{51}Cr activity was assessed by multiplying the supernatant aliquot count by a factor of 2.5 (200 µl aliquot from a total of 500 µl culture) by 1.5 (100 µl aliquots from a total of 150 µl culture), or 2.0 (100 µl aliquots from a total of 200 µl culture). In early experiments, the cell pellets were also counted in parallel so that the total radio-activity per given number of target cells could also be assessed, but it was found that this method gave no better results than those obtained by counting a number of "blanks" containing aliquots of the appropriate number of target cells which were counted

in toto.

Spontaneous release controls and maximum release controls were incorporated with every experiment and the degree of specific lysis of antibody sensitised red cells was calculated and expressed as percent specific lysis as shown below.

$$\% \text{ specific lysis} = \frac{\% \ ^{51}Cr \text{ release in test}^{*} - \% \ ^{51}Cr \text{ spont. release}^{**}}{\% \text{ maximum } ^{51}Cr \text{ release}^{***} - \% \ ^{51}Cr \text{ spont. release}} \times 100\%$$

* Effector cells + RBC (with or without anti-D)

** RBC + TC 199 (or anti-D, inhibitors etc. - see text)

*** RBC + distilled H_2O or 1% saponin

The spontaneous ^{51}Cr release was usually similar in TC 199 alone or with anti-D containing material, in which case the average was used for calculations; if the spontaneous release was significantly higher in the presence of any material other than TC 199, the higher value was used in the calculation of % specific lysis.

9.6 Estimation of RBC phagocytosis

Since there were too few cells under the culture conditions above to estimate phagocytosis on morphological grounds, this was done on the basis of ^{51}Cr uptake by the effector cell population. Replicate assays were set up as above for the K-cell assay and after the appropriate incubation time, the microplates were centrifuged at 200 g for 10 minutes and supernatants removed for ^{51}Cr counting as above. The cell pellet was then resuspended in hypotonic lysis medium, centrifuged at $4^{o}C$ for 5 minutes at 200 g, the supernatant removed and the

process repeated. The residual cell pellets were then washed in TC 199 3 times, resuspended in 200 µl TC 199, and the total amount removed for ^{51}Cr estimation. Intracellular ^{51}Cr was taken to represent ingested RBC and expressed as a percentage of the original ^{51}Cr per RBC. Control cultures were set up in parallel with ^{51}Cr labelled RBC without anti-D to assess non-specific ^{51}Cr uptake by mononuclear cells during the procedure.

10.0 PREPARATION OF HUMAN IMMUNOGLOBULINS

10.1 Preparation of immunoglobulin G (IgG)

The starting material was pooled normal human IgG batch no. 208 prepared by the Scottish National Protein Fractionation Centre for human use. 5 ml (750 mg/ml) was dialysed overnight against TC 199 at 4°C. This material was then diluted in TC 199 to a concentration of 20 mg/ml. The IgG contained some contaminating IgA and IgM and this was removed by passage through immunoadsorbent columns of Sepharose-anti-IgA and Sepharose-anti-IgM prepared as described below (section 11.0). The purity was checked by immunodiffusion against ultra-low level plates (Hyland) and aliquots stored at -40°C until required. Dilutions were made in serum-free TC 199 to give the required concentrations for inhibition assays (see below).

10.1a Preparation of IgG_1, IgG_2 and IgG_4

IgG of known sub-classes were obtained from myeloma patients with high levels of the abnormal paraprotein (at least 30 mg/ml). Two samples of IgG_2 and one of IgG_3 were a gift from Dr. J. Wallace and Mr. G. Templeton, West of Scotland Blood Transfusion Service. The IgG_1, IgG_2 and IgG_4 components were isolated from serum or plasma samples using an affinity column of Staphyloccal protein A-Sepharose (Pharmacia) according to the method of Skvaril, (1976). IgG subclasses of 1, 2 and 4 bind specifically to this material and the separated proteins can then be eluted in a purified form.

5 mg of gel was pre-swollen according to the manufacturers instructions using 0.1 M phosphate buffer pH 7.5. The gel was packed into a glass fractionation column and washed with ice-cold phosphate buffer, pH 7.5. The capacity of a column containing 5 mg of protein A-Sepharose is approximately 120 mg of IgG, and serum or plasma aliquots were added to the column in such a volume as to contain approximately 100 mg IgG. Elution was performed in the cold-room at 4°C and unbound proteins were eluted in approximately 20 mls 0.1 M phosphate buffer pH 7.5 and 1 ml fractions collected in glass tubes. The IgG was then desorbed using 0.2 M glycine-HCl pH 3.0 containing neutral red as a marker, at a rate of 1 ml per minute and 1 ml fractions collected as above. The column was then washed with a further 20 mls phosphate buffer. The front of the IgG elution was indicated by the neutral-red marker and these fractions (approximately 25 x 1 ml) were neutralised by the addition of 50 µl 1 M Tris pH 10. The exact position of the IgG protein peak was then found by measuring the absorbance at 280 nM in a spectrophotometer (Cecil). The fractions containing the major protein peak were then pooled (approximately 10 x 1 ml fractions). This protein peak contains pure IgG and this was tested for contaminating IgA or IgM in ultra-low immunodiffusion plates (Hyland).

The IgG was concentrated by ultra-filtration, followed by equilibration against TC 199 by dialysis overnight at 4°C, and aliquots stored at -40°C until required.

Dilutions were made in serum-free TC 199 to give the required concentrations for inhibition assays.

10.1b Preparation of IgG_3

The IgG_3 subclass does not bind to Staphyloccal protein A and therefore cannot be separated by the above method. 5 ml plasma containing approximately 60 mg/ml IgG_3 was used as the starting material for the preparation of an IgG fraction by the method described in section 8.1b.

The final concentrate was dialysed against TC 199 at 4°C overnight, and aliquots stored at -40°C until required. Dilutions were made in serum-free TC 199 to give the required concentration for inhibition assays.

10.2 Preparation of immunoglobulin A (IgA)

This was prepared from a serum of a patient with IgA myeloma according to the method of Fine & Steinbuch (1970) by Dr. D.S. Pepper.

10 mls of serum containing approximately 60 mg/ml IgA was placed in a beaker and 4 g ammonium sulphate added with continuous stirring to give a solution containing 40% ammonium sulphate. The IgG/IgA precipitate was recovered by centrifugation, washed with 40% ammonium sulphate, recentrifuged and the precipitate redissolved in 10 mls normal saline and dialysed overnight against saline. 20 mls 0.06 M sodium acetate buffer pH 4.8 were added, followed by 10 mls caprylic acid (BDH), with continuous mixing. The clear supernatant containing IgG/IgA was collected following centrifugation, filtered, dialysed overnight against normal saline at pH 8, followed

by further dialysis at 4°C against 0.015 M sodium acetate buffer, pH 7.5. Twenty five g. dried DEAE cellulose (Whatman) was equilibrated with the same buffer and slowly added, with stirring, to the IgA/IgG solution. The IgA remains bound to DEAE cellulose whereas the IgG is not. The gel was washed with acetate buffer on a Buchner filter and the IgA eluted with 0.09 M acetate buffer pH 5.7. The eluate was passed through Sepharose anti-IgG and anti-IgM columns to remove contaminating IgG and IgM (see section 11.0) and the purity checked by immunodiffusion against ultra-low level plates (Hyland). IgA was then concentrated by ultra-filtration followed by dialysis against TC 199 and aliquots stored at -40°C until required. Aliquots were diluted in serum-free TC 199 to give the required concentration in culture.

10.3 Preparation of Immunoglobulin M (IgM)

IgM was obtained from plasma of a patient with Waldenstrom's macroglobulinaemia with an IgM level of approximately 100 mg/ml. Purified IgM was obtained using a protamine-Sepharose affinity column by the method of Wichman & Borg (1977) and was kindly done by Dr. D.S. Pepper. Protamine was coupled to Sepharose 4B (Pharmacia) by the cyanogen bromide method (Cuatrecasas, 1970). The coupled gel was then equilibrated with 0.08 M phosphate buffer pH 7.4. Five mls of the gel and 10 mls of distilled water were added to 10 mls of plasma, followed by stirring for 3 hours at 4°C. The absorbed gel containing the IgM was washed with diluted phosphate buffer (1 vol. buffer, 2 vols. distilled water) on a Buchner filter and packed into a glass fractionation

column. Unbound proteins were eluted with diluted phosphate buffer and the IgM then eluted with diluted buffer containing 1.1 M sodium chloride. 1 ml fractions were collected and the IgM-containing fractions were identified by gel diffusion against anti-IgM (Hyland plates). The IgM fractions were then pooled and concentrated by ultra-filtration. Contaminating IgA and IgG was removed by passage through Sepharose-anti-IgG and Sepharose anti-IgA columns (see section 11.0) and the purity checked against ultra-low level immunodiffusion plates (Hyland). The purified IgM was then dialysed overnight against TC 199 and stored in aliquots at -40°C until required and diluted in serum-free TC 199 for inhibition assays.

10.4 Inhibition assays with immunoglobulins

Pooled normal IgG, IgA, IgM, and IgG subclasses as prepared above were diluted in serum free TC 199 and ultracentrifuged at 40,000 g immediately before use in the K-cell assay. The desired concentration in 150 µl or 200 µl cultures was achieved by adding 50 µl or 100 µl of immunoglobulin solution. To avoid the addition of extraneous human immunoglobulin, the effector cells were suspended in TC 199/10% FCS, and the RBC were presensitised with anti-D and suspended in the same culture medium. The E/T was usually 10:1 with 18 hr. cultures set up with appropriate controls as described in section 9.3.

11.0 PREPARATION OF IMMUNOADSORBENT COLUMNS

CN.Br.-activated Sepharose was obtained from Pharmacia and prepared according to the manufacturers instructions. 5 g gel was swollen with 0.001 M HCl followed by washing in a Buchner filter with the same solution and divided into 3 aliquots. To the first was added 1 ml rabbit anti-IgG (gamma-chain specific) (Behringwerke), to the second, 1 ml rabbit anti-human IgA (alpha-chain specific) (Behringwerke) and to the third, 1 ml rabbit anti-human IgM (mu-chain specific) (Behringwerke). To each aliquot was added 5 ml 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl. The gels were and rotated end-over-end in glass test tubes for 2 hours at room temperature.

Unbound material was eluted with bicarbonate buffer and the gel deactivated by the addition of 5 ml 1 M ethanolamine at pH 8 for 1 hour. The gels were then washed to remove non-covalently bound material with 3 washing cycles consisting of 1) 0.1 M acetate buffer containing 0.5 M NaCl at pH 4 and 2) 0.1 M borate buffer containing 0.5 M NaCl at pH 8. The gels were then stored at $4^{o}C$ in saline containing 1% sodium azide until used.

For use, columns were prepared by packing the gels into 10 ml sterile syringes containing a nylon wool pad and fitted with a 3-way tap. After elution with PBS pH 7.3 and TC 199, the solutions to be adsorbed were added to the columns, allowed to adsorb at room temperature for 30 minutes, and eluted with TC 199.

The eluates were then tested and concentrated as described in section 10.0.

12.0 PREPARATION OF AGGREGATED POOLED NORMAL IgG

Pooled normal IgG was prepared as above (para. 10.1) and then divided into 2 aliquots of 2.5 mls each, one of which was used to prepare heat-aggregated material and the other was used as the normal unaggregated control.

2.5 mls were heat-aggregated at 63°C for 30 minutes (Dickler & Kunkel 1972). The solid aggregates were then redissolved in 5 mls TC 199, centrifuged at 150 g for 10 minutes to remove large aggregates and the supernatant removed. The protein concentration of this material was 9.5 mg/ml and it was stored at -40°C in aliquots until required.

12.1 Inhibition assays with aggregated IgG

a) Aliquots of effector cells (3×10^6), prepared as above following nylon column passage (para. 3.2a) were incubated at 37°C for 30 minutes with aggregated and non-aggregated pooled IgG (prepared as above) at concentrations from 5 to 5000 µg/ml of IgG. After incubation, 10 mls of medium 199 was added and the effector cells were washed twice at 200 g before being resuspended in TC 199/10% FCS (ie. no human IgG).

Anti-D pre-sensitised RBC were prepared as above (6.3) but were finally resuspended in medium 199/10% FCS.

b) Aggregated and non-aggregated IgG as prepared above was added directly to cultures so that it was present throughout the incubation period. Again, the culture medium was supplemented with FCS.

13.0 PREPARATION OF METABOLIC INHIBITORS

13.0.1 2-deoxyglucose (Sigma)

164.2 mg crystals (molecular weight 164.2) was dissolved in 10 mls TC 199 to give stock solution of 100 mM. This was diluted in TC 199/10% AB serum to give a final range of concentrations in culture of 1-50 mM. K-cell cultures were performed as described in para. 9.3 with 50 µl of RBC suspension, 50 µl of effector cell suspension and either 50 or 100 µl of the appropriate dilution of 3-deoxyglucose to give the final concentrations as stated above.

13.0.2 Mitomycin C (Sigma)

2 mg freeze dried powder prepared from Streptomyces caespitosus was dissolved in 2 ml TC 199 giving a stock solution of 1000 µg/ml. For inhibition assays, dilutions in TC 199/10% AB serum were prepared as above to give a final range of concentrations of 5-25 µg/ml.

13.0.3 Actinomycin D (Sigma)

1 mg dried powder prepared from Actinomyces antibioticus was dissolved in 10 ml TC 199 giving a stock solution of 100 µg/ml containing antibiotics. Appropriate dilutions in TC 199/10% AB serum were prepared as above to give a final range of concentrations of 0.5-10 µg/ml.

13.0.4 Puromycin (Sigma)

1 mg crystals was dissolved in 10 ml TC 199 giving a stock solution of 100 µg/ml. For dilutions TC 199 were prepared as above to give a range of concentrations of 0.5-10 µg/ml.

13.0.5 Colchicine (Sigma)

100 mg 39.94 mg dried powder (molecular weight 339.4) was dissolved in 10 ml TC 199 giving a stock solution of 10 mM. Appropriate dilutions in TC 199/ 10% AB serum were made as above giving a final range of concentrations of 0.1-5 mmols.

13.0.6 Hydrocortisone (Organon Laboratories Ltd)

100 mg freeze-dried hydrocortisone sodium succinate was reconstituted in 20 mls TC 199 to give a stock solution of 10 mM hydrocortisone sodium succinate (molecular weight 502.56) or 13.8 mM hydrocortisone (molecular weight 362.47). This was diluted as required in TC 199/10% AB serum to give a final range of concentrations of 0.1-5 mM hydrocortisone sodium succinate (equivalent to 0.139-6.9 mM hydrocortisone) as above.

13.0.7 Cytochalasin B (Aldrich Chemicals, I.C.I.)

A stock solution containing 50 µg/ml was made by dissolving 5 mg per 100ml sterile saline (0.9%) containing 0.2% dimethylsulphoxide (DMSO). Dilutions of the stock material were made in TC 199/10% AB serum as above to give a final range of concentrations of 0.025-5 µg/ml.

13.1 Controls for the inhibitor assays

With each inhibitor as prepared above, at least the highest concentration, and more usually, the whole range of concentrations were tested against the target RBC and against the mononuclear cell suspensions alone

in culture in order to assess any cytotoxic effects. The above range of concentrations were chosen as the highest concentration of inhibitor present during 18 hour culture which did not effect the viability of the effector cell population or result in target cell damage as shown by an increase in the spontaneous ^{51}Cr release.

13.2 Calculation of degree of inhibition

With each of the metabolic inhibitors, the effects of the various concentrations were compared with replicate control cultures without any inhibitor present. Because of variation in the % specific lysis between individuals and with the same individuals tested on different days, the results were "normalised" by being expressed as a percent cytotoxic activity taking the uninhibited culture as the 100% value according to the formula:

$$\% \text{ cytotoxic activity} = \frac{\% \text{ specific lysis with inhibitor}}{\% \text{ S.L. without inhibitor}} \times 100\%$$

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1.0 EFFECT OF PAPAINISATION OF TARGET RBC

In early experiments it was found that lysis could be obtained using human lymphoid cells and D positive RBC in the presence of high titre anti-D. However, it proved difficult to obtain in consistently high levels of ^{51}Cr release as obtained, for example, with chicken RBC or sheep RBC (Calder et al 1974, Perlmann et al 1972).

It was therefore decided to attempt enhancement of lysis by the proteolytic enzyme treatment of RBC. This method had been used with success by Holm (1972) with monocyte lysis of human RBC and the use of such enzymes to enhance antibody-red cell interactions is well documented in red cell serology and blood transfusion practice (Mollison 1972) especially with antibodies of the Rhesus blood group.

The effect of papain pre-treatment of RBC before addition to ADCC cultures is shown in Table 1.0.1. It can be seen that there is significant enhancement of lysis with a variety of D positive RBC types in the presence of anti-D from several sources.

The enhancement of lysis is even more marked at low effector/target (E/T) ratios where there are probably only very small numbers of effector cells present in culture (table 1.0.2).

In table 1.0.3, the enhancement of specific lysis is quite considerable, especially with serum (2) where there is no lysis with untreated RBC but 39.7% specific lysis (S.L.) with papainised RBC after monocyte removal. A further significant feature is the increase in specific lysis noted following monocyte removal; these findings

are investigated further in para. 5.0 and section III but it would appear that there is selective enrichment of a non-monocytic K-cell.

There are several possible reasons for the mechanism of enhancement. It is thought that one of the actions of the proteolytic enzymes on RBC membranes is to increase the amount of antibody uptake (Masouredis 1962; Hughes-Jones, 1964). Increased antibody binding would result in the presence of more activated Fc antibody determinants being available for the initiation of the K-cell lytic process. Further evidence for the dosage effect of antibody is presented in para. 6.0. It is also possible that the removal of surface glyco-proteins allows better cell-to-cell contact with the Fc portion of the antibody molecule being more accessible. The rhesus antigen is thought to be part of the lipoprotein membrane of the RBC (Green 1972) "buried" between the glycoprotein chains bearing other RBC antigens such as A, B, H., M, N, S, and this is thought to be one of the reasons why rhesus antibody molecules do not normally fix complement although of the appropriate IgG subclass (Mollison 1972). It has also been reported that enzymic action on the RBC membrane allows movement of the antigen sites in the membrane which is in a semi-fluid state (Voak et al 1974; Romano et al 1975) and thus cell-bound antibody molecules with activated Fc determinants may form "clusters" allowing a greater density of activated Fc determinants to be presented to the K-cell.

Papainisation may also render RBC more "labile" and more susceptible to lysis since enzyme treated RBC will not survive in vitro for long as the native RBC under the same conditions. However, under the conditions of the ADCC culture of 18 hours at 37°C the amount of spontaneous ^{51}Cr release is not significantly different for papainised or untreated RBC (see table 1.0.4). It can also be seen from these results that TC 199 alone gives in general higher spontaneous release than does medium supported with additional protein either as AB serum or anti-D containing serum to equivalent concentration. It can also be seen that the presence of anti-D does not increase the spontaneous ^{51}Cr release with either papainised or untreated RBC - usually the opposite in fact, which suggests that the presence of anti-D on the cell surface alters the membranes some undefinable way. Completely serum-free culture medium gave very high spontaneous ^{51}Cr releases (table 1.0.5).

Papainised RBC will spontaneously agglutinate in the presence of anti-D after settling in culture and it is possible that this contributes to the enhancement of the lytic process by K-cells. Agglutination of the sensitised RBC cells by anti-D per se does not appear to effect the spontaneous ^{51}Cr release as seen in table 1.0.4.

Conclusions: Papainisation of red cells increased the specific lysis by K-cells without significantly increasing spontaneous ^{51}Cr release during culture and therefore produced a system of greater sensitivity for the assessment

of the in vitro characteristics of this particular K-cell assay.

TC 199 supplemented with serum was required to maintain an acceptable spontaneous ^{51}Cr release from RBC and human group AB serum was used for most cultures.

Because of the significant enhancement afforded by papainisation, all further experiments (unless stated to the contrary) involved the use of papainised RBC targets.

Table 1.0.1 PAPAINISATION OF RBC - ENHANCEMENT OF SPECIFIC LYSIS

	E/T ratio*	Lym. donor group	Target group	anti-D** serum source	% specific lysis			
					untreated RBC		papainised RBC	
					+anti-D	+AB serum***	+anti-D	+AB serum
1.	5:1	AB R_1R_1	O Ror	BTS 103 1/10 free	38.3	-10.7	64.0	2.3
2.	10:1	AB R_1R_1	O R_1R_2	Cumming 1/10 presens.	24.6	-1.3	70.0	1.5
3.	10:1	O rr	O R_1R_2	Louden IgG II 1/30 free	24.0	-5.6	30.9	0
4.	10:1	A R_1R_1	O R_1R_1	Louden 1/6 free	24.0	NT	95.0	NT

* Effector/target cell ratio (10^5 RBC except 4. (0.4×10^5 RBC))

** Dilution of anti-D in culture or used to pre-sensitise RBC prior to culture

*** AB serum diluted to equivalent of anti-D serum

NT Not tested

Adherent monocytes removed; 19 hr culture

Table 1.0.2 PAPAINISATION OF RBC - ENHANCEMENT OF SPECIFIC LYSIS AT VARIOUS EFFECTOR/TARGET RATIOS

	% specific lysis with			
	Untreated RBC		Papainised RBC	
	+ anti-D*	+ AB serum	+ anti-D*	+ AB serum
10:1	24.0	-5.6	30.9	0
5:1	6.0	-8.9	21.2	-8.2
1:1	0	2.9	15.5	-4.0
1:5	-4.4	-2.5	10.5	-5.1

* IgG I anti-D; final dilution 1/30 free in culture
Lym. donor O rr : RBC donor O R_1R_2 (1×10^5 per culture)
adherent monocytes removed ; 18 hr culture

Table 1.0.3 EFFECT OF RBC PAPAINISATION PRE AND POST MONOCYTE REMOVAL

	% specific lysis with			
	untreated RBC		papainised RBC	
	+anti-D	+AB serum	+anti-D	+AB serum
serum (1)				
pre nylon	14.7	2.0	27.8	0.5
post nylon	15.5	1.7	56.7	2.2
serum (2)				
pre nylon	0.2	0.5	10.2	1.0
post nylon	1.6	-0.2	39.7	-5.2

(E/T 10:1 (4×10^4 RBC)

serum (1) Louden anti-D serum 1/3 free in culture

serum (2) Campbell serum (anti-D papain titre 1/128)

18 hr. cultures

Table 1.0.4 SPONTANEOUS RELEASE OF ^{51}Cr. DURING CULTURE (18 hr) PAPAINISED RBC VS. UNTREATED RBC

RBC type	incubation medium	papainised % ^{51}Cr release****	untreated % ^{51}Cr release
1. O R_1R_2	199+	12.5	10.3
2. O R_1R_2	199	2.9	3.4
3. O R_1R_1	199	15.4	13.0
	anti-D/199*	2.3	4.0
4. O R_1R_1	AB/199**	5.5	6.0
	anti-D/199***	4.7	3.9
5. O R_1R_1	AB/199	7.3	4.1
	anti-D/199***	6.2	2.2
	mean	7.1	5.9
	S.D.	4.6	3.8

* Louden serum : final dilution 1/3

** AB serum : final dilution 1/3

*** Louden IgG I anti-D : final dilution 1/120

**** expressed as % of total available ^{51}Cr in RBC pre-incubation

\+ 199 supplemented with 10% AB serum

No significant difference between means (paired t test)

Table 1.0.5 SPONTANEOUS ^{51}Cr RELEASE - PROTECTIVE EFFECT OF SERUM

	RBC % ^{51}Cr release over 18 hr culture in		
	100% AB serum	10% AB serum/199	199
$4x10^4$ RBC	15.2	10.2	78.6
$4x10^4$ RBC $+$ $4x10^5$ Effectors	17.4	14.1	71.5

2.0 EFFECT OF PAPAINISATION ON EFFECTOR CELLS

It has been reported that treatment of K-cell populations with proteolytic enzymes results in increased cytotoxic activity (Kedar et al 1974), although these findings are not universally confirmed (Werner et al 1976). It has also been reported that papainisation of monocytes enhances the binding of RBC sensitised with IgG antibody (Kenna et al 1975).

Hence the effect of papainisation on the effector K-cell population was investigated and the results are shown in table 2.0. It can be seen that papainisation of the effector cell population reduced the % specific lysis considerably (42.1% to 17.1% S.L.) whilst the REC target, in the absence of anti-D, is not lysed by either effector cell population. The viability of the effectors was not reduced by enzyme treatment.

Conclusion: It is therefore most unlikely that the enhancement of RBC lysis by papainisation of the target RBC is due to inadvertent papainisation of the effector cell, due either to accidental "carry-over" of enzyme on the RBC surface or due to inadequate washing.

Table 2.0 PAPAINISATION OF EFFECTOR CELLS

	% S.L.
untreated effectors + anti-D + RBC	42.1
" " + AB serum + RBC	-11.5
papainised effectors + anti-D + RBC	17.1
" " + AB serum + RBC	-6.9

Lym. donor O R_1R_1. Anti-D (Louden) 1/3 final concentration

RBC donor O R_1R_1. E/T 10:1. 18 hr. culture

Adherent monocytes removed

3.0 VARIATION IN K-CELL LYSIS DUE TO EXPERIMENTAL AND METHODOLOGICAL ERRORS

In a biological system there is inevitable variability when comparing one individual with another, and the same individual on different occasions (see chapt. IV, para. 3.0). It is important to exclude, or at least minimise, the errors and variability due to the technique itself.

It is also important to know the magnitude of these errors if one is to make meaningful interpretations of differences observed during the various *in vitro* investigations into the nature of the K-cell mechanism.

The methodological variations were assessed in two ways.

3.1 Variation between replicates

Effector cells and RBC were prepared from two donors and replicate (x 10) cultures were set up from each. These cultures were then processed separately as if from two lots of 10 different individuals. The results are shown in table 3.1 where it can be seen that the coefficient of variation is well below 10% for both individuals (8.7% and 6.3% respectively) and this was considered an acceptable degree of variation for a biological system.

3.2 Variation in separation technique

60 mls of blood from one individual was divided into 6 x 10 ml aliquots which were processed independently throughout the separation procedure and culture assay as if from 6 different individuals. The following points emerged (table 3.2).

1. The coefficient of variation is comparable to that of the previous experiment (7%) and therefore the separation technique itself does not give rise to unduly large discrepancies between individuals who might otherwise have displayed similar K-cell activity. This means that one can have reasonable confidence that differences seen between individuals are "real".

2. The nylon wool adherence method is also reproducible and the recovery of white cells and degree of monocyte removal is similar in all cases. This experiment also demonstrates the efficiency of nylon wool columns in removing adherent cells.

3. As noted in para. 1.0, K-cell activity against unsensitised RBC (ie. in AB serum) is insignificant compared to the degree of lysis in the presence of the appropriate antibody. The large coefficient variation seen in table 3.2 with AB serum (26.5%) is largely due to the individual variations seen with very low levels of specific lysis.

In conclusion the coefficients of variation induced by the various <u>in vitro</u> manipulations are of the order of 10% indicating that differences between individuals larger than 10% are likely to be significant.

Table 3.1 COEFFICIENT OF VARIATION OF REPLICATES

Replicate	donor (1) % specific lysis	donor (2) % specific lysis
1.	68.7	60.4
2.	81.2	61.3
3.	76.8	62.3
4.	78.9	60.9
5.	62.9	58.1
6.	68.4	60.2
7.	63.2	68.9
8.	70.5	64.6
9.	69.2	65.8
10.	73.5	70.1
mean	71.3	63.3
N	10	10
S.D.	6.2	4.0
V*	8.7	6.3

Lym. donor (1) : 0 R_1R_1 RBC donor : 0 R_1R_1

Lym. donor (2) : 0 R_1r

E/T 10:1 (1 x 10^4 RBC per culture)

Louden IgG I anti-D final dilution 1/120 in culture

(18 hr.) : adherent monocytes removed

* Coefficient of variation

Table 3.2 COEFFICIENT OF VARIATION OF SEPARATION TECHNIQUES

Aliquot	% monocytes*		% recovery** of lymphoid cells	% specific lysis	
	pre-nylon	post-nylon		+ anti-D	+AB serum
1.	5.0	1.0	69.6	57.7	5.1
2.	6.0	0.8	64.7	64.0	6.9
3.	5.0	1.0	67.2	62.8	8.5
4.	4.0	1.5	54.5	66.3	6.7
5.	6.0	0	60.7	66.0	8.6
6.	6.0	0	45.8	71.4	11.1
mean	5.3	0.7	60.4	64.7	7.8
S.D.	0.8	0.6	8.9	4.5	2.1
V	-	-	14.8	7.0	26.5

* estimated by phase contrast

** estimated from differential white cell count prior to separation
Lym. donor A R_1R_1 : RBC donor O R_1R_1 : E/T 10/1 (1×10^4 RBC)
Louden anti-D serum final dilution 1/6 in culture (18 hr); adherent monocytes removed

4.0 EFFECT OF ALTERATIONS IN CULTURE CONDITIONS

4.1 "macrotube" assays compared with "microplate" assays

As noted in chapt. III, para. 9.0 the conditions for K-cell assay changed from macrotube to microplate. The majority of experiments were done at a fixed effector/target cell ratio of 10:1 in the presence of an excess (see para. 6.0) of anti-D antibody whilst the behaviour of other variables was investigated.

The results in table 4.1 demonstrate that the % S.L. obtained with the macrotube and microplate assays, (other conditions being similar) are in very close agreement - mean 66.1 ± 0.3% S.L. for macrotube assays and 61.7 ± 4.2% S.L. with the microplate assays. The difference between the paired means is not statistically significant. It is interesting that these donors have shown a similar % S.L. despite being studied at two different times some months apart.

4.2 Alteration of culture volume

In certain series of experiments the effects of some inhibitors were studied in 200 µl culture volume, and others in 150 µl volume. The results in table 4.2 show that with the same effector cell, target cell and anti-D sources, the final % S.L. achieved is very similar despite the fact that there is slightly greater dilution of anti-D in the larger culture volume - this indicates that the antibody is present in excess at both concentrations. The differences in means, 58.9 ± 16.2 % S.L. with 200 µl cultures and 55.8 ± 15.1% S.L. with 150 µl

cultures, are not statistically significant.

In experiment 1. the amount of anti-D used to pre-sensitise the RBC was identical for both culture volume assays and it can be seen that a similar % S.L. was attained.

4.3 Increasing absolute numbers of effector and target cells at constant E/T ratio and increased culture volume

The results shown in table 4.3 indicate that provided the effector/target cell ratio is constant (at 10:1) one can expect a similar final % specific lysis if cells and antibody are from the same source. In this particular experiment, the culture volume was also increased so the effectors and targets are at the same density despite the increase in volume.

4.4 Increasing absolute number of effector and target cells at constant E/T ratio with constant culture volume

In this particular experiment cells from the same donors were used throughout. Again, it can be seen in table 4.4 that provided the E/T is maintained at 10/1, the final % specific lysis are not statistically significantly different. It was apparent however, that a lower cell density (experiment 1 and 2) was slightly detrimental to the spontaneous ^{51}Cr release.

4.5 Conclusions

The K-cell assay is very robust in that under conditions of antibody excess the limiting factor in achieving lysis appears to be the relative number of effector and target cells in culture ie. the E/T ratio.

Alteration in methodology which results in different culture volumes and cell densities does not appear to significantly affect the end-point of specific lysis by effector cells.

Table 4.1 "MACROTUBE" VS. "MICROPLATE" ASSAYS

Lym. donor	E/T	anti-D** donor	% S.L. +anti-D	% S.L.*** +AB serum	Final culture volume
1. B rr	A. 10/1	IgG I 1/120 free	66.8	3.6	600
	B. 10/1	IgG I 1/120 presens	57.7	3.9	150
2. O R_1R_1	A. 10/1	Louden 1/6 free	66.4	2.2	600
	B. 10/1	Louden 1/3 free	66.1	1.6	150
3. B R_1R_1	A. 10/1	IgG I 1/120 free	65.8	3.9	600
	B. 10/1	IgG I 1/100 free	61.4	NT	150
		600 µl mean	66.1	3.2	
		S.D.	0.3	0.9	
		150 µl mean	61.7	3.2	
		S.D.	4.2	1.6	

Same lym. donors studied on 2 separate occasions: A(macrotube assay) and B (microplate assay)

* E/T = effector:target cell ratio A : 1×10^5 RBC B : 0.4×10^5 RBC

** final concentration of anti-D in culture: O R_1R_1 RBC targets on each occasion : adherent cells removed

*** percent specific lysis of RBC in presence of anti-D (as shown), or AB serum during 18 hr. culture

Paired t test for difference between means - not significant

Table 4.2 EFFECT OF ALTERATIONS IN CULTURE VOLUME

Lym. donor	E/T	anti-D donor	% S.L. +anti-D	Final culture volume (µl)
1. O R_1r	B1. 10/1	IgG II presens 1/2	36.1	200
	B2. 10/1	IgG II presens 1/2	37.6	150
2. B rr	B1. 10/1	IgG III 1/40 free	74.1	200
	B2. 10/1	IgG III 1/30 free	74.6	150
3. O R_1r	B1. 10/1	IgG III 1/4 free	64.0	200
	B2 10/1	IgG III 1/3 free	55.1	150
4. O R_1r	B1. 10/1	Louden 1/4 free	61.7	200
	B2. 10/1	Louden 1/3 free	55.8	150
		200 µl mean	58.9	
		S.D.	16.2	
		150 µl mean	55.8	
		S.D.	15.1	

B1. and B2. performed simultaneously under similar conditions (0.4×10^5 O R_1R_1 RBC per culture) apart from culture volume.

Microplate assays : details as for table 4.1

Paired t test for difference between means - not significant

Table 4.3 EFFECT ON INCREASING ABSOLUTE NUMBER OF EFFECTOR AND TARGET CELLS WITH CONSTANT RATIO AT 10/1 AND INCREASING VOLUME

Lym. donor	E/T	anti-D donor	% S.L. +anti-D	% S.L. +AB serum	Final culture volume (µl)
B rr	C. 10/1	Louden 1/3 free	44.3	NT	600
	D. 10/1	Louden 1/3 free	46.6	NT	150

donor (1), table 4.1, on a different occasion. C : 1.6×10^5 RBC
D : 0.4×10^5 RBC (O R_1R_1 donor). Microplate assays; both experiments done simultaneously with different culture volumes. Details as for table 4.1.

Table 4.4 EFFECT OF INCREASING ABSOLUTE NUMBER OF EFFECTOR TARGET CELLS AT CONSTANT RATIO AT 10/1 WITH CONSTANT CULTURE VOLUME

	Effectors x 10^4	RBC* x 10^4	% ^{51}Cr spont. release	% ^{51}Cr max. release	% S.L.
1.	10	1	10.9	100	52.0
2.	20	2	11.9	100	49.0
3.	40	4	9	100	61.3

* O R_1R_1 pre-sensitised with Louden serum (neat)

Details as for table 4.1. Microplate cultures with constant volume at 150 µl

5.0 ALTERATION OF EFFECTOR/TARGET CELL RATIOS AT FIXED ANTI-D CONCENTRATION

As indicated in para. 4.0, it appears that the limiting factor in terms of specific lysis of sensitised RBC is the relative number of effector and target cells in culture provided that there is an excess of sensitising antibody. The number of effector cells is likely to remain constant during the relatively short culture incubation period of 18 hrs. and therefore when the numbers of mononuclear cells are changed relative to a given number of RBC, the % specific lysis obtained is a reflection of the number of effector K-cells present.

5.1 Removal of monocytes on plastic dishes

This is illustrated in fig. 5.1 where the data from eight individuals have been analysed. The anti-D concentration was chosen as that dilution (1/120) which gave maximal % specific lysis at an E/T of 40:1 and anti-D was therefore assumed present in excess. In this series of experiments monocytes were removed by plastic dish adherence (chapt. III, para. 3.1) reducing the mean % monocytes to 2.5%.

It can be seen that a sigmoidal dose-response type of curve is obtained from the mean % S.L. at each E/T ratio for the eight individuals. There is a relatively linear increase in % S.L. with increasing numbers of effector cells over the range of E/T ratios of 1:1 up to 20:1 (1×10^4 - 20×10^4 mononuclear cells per culture). Very little lysis is seen below 1:1 where presumably the effector cells are unable to lyse sufficient numbers of RBC to give ^{51}Cr release above background spontaneous

release. A plateau effect begins to appear after 10:1 to 20:1 with maximum % S.L. in the region of 80-90% - the increase at 40:1 is largely due to a few high values in a small series of observations. The effect of an equivalent number of effector and target cells in culture with RBC antibody-free AB serum is also illustrated in fig. 5.1 and it can be seen that unsensitised red cells are not lysed above background even at the highest effector to target cell ratio of 40:1 indicating that there is no direct toxicity to RBC in the absence of the appropriate antibody.

Examination of the data in table 5.1 shows that there is a considerable degree of individual variation in the point at which the maximum % S.L. is seen - donors 1 and 5 achieved 96.7% and 100% S.L. at 10:1 whereas donors 2 and 4 achieved only 28.9% and 47.7% respectively.

The number of effector cells per culture is unknown since there is no way of detecting which of the mononuclear cells are the active K-cells and when one is measuring a functional end point ie. lysis, one does not know whether increased lysis is due to an increased number of effector cells or an increase in efficiency of a small number of cells.

In order to standardise the data from a series of individuals for comparision it is possible to calculate the numbers of mononuclar effector cells required to produce 50% S.L. from the dose response curve as in fig. 5.1 - in this case 6×10^4 effectors per 10^4 RBC.

Table 5.1 EFFECT OF ALTERING EFFECTOR CELL/TARGET RBC RATIO AT FIXED ANTI-D CONCENTRATION

Lymph. donor group	%*** monos	Effector - target ratio									
		1/10 *0.1	1/5 0.2	1/2.5 0.4	1/1 1.0	2.5/1 2.5	5/1 5.0	10/1 10	20/1 20	30/1 30	40/1 40
1. O R_1r	1.0	**3.9	3.8	-	11.2	-	16.9	96.7	97.8	67.5	-
2. O R_1R_2	4.0	18.1	15.9	5.6	18.2	23.5	22.9	28.9	61.4	64.9	-
3. O R_1R_1	0	8.3	3.8	12.6	24.5	24.3	54.8	83.5	98.8	92.8	-
4. AB R_1R_1	4.0	18.3	17.2	10.3	15.7	27.1	29.9	47.7	54.2	65.6	85.2
5. AB R_1R_1	4.0	6.3	18.9	21.2	16.9	39.6	67.8	102	96.5	86.6	94.1
6. B R_1R_1	4.0	-6.4	-3.9	0	0	22.5	21.6	65.8	82.2	108.1	90.4
7. B rr	2.0	4.3	2.5	2.9	12.6	25.4	38.0	66.8	67.2	61.2	80.2
8. A R_1R_2	1.0	-2.4	-5.3	2.4	6.0	37.1	66.7	79.5	95.4	101.7	99.7
mean	2.5	6.3	6.6	7.9	13.1	28.5	41.1	71.4	81.7	81.1	89.9
S.D.		8.73	9.52	7.39	7.55	6.92	19.6	24.52	18.27	18.53	7.85
N		8	8	7	8	7	8	8	8	8	5
SEM		3.09	3.37	2.79	2.67	2.62	6.93	8.67	6.46	6.55	3.39

* x 10^4 effectors per culture (1 x 10^4 O R_1R_1 RBC per culture : microtube assay)

** % specific lysis

*** estimated by phase contrast after plastic incubation

anti-D IgG I 1/120 dilution in all cultures

18 hr. culture : final vol. 500 µl : adherent monocytes removed (plastic dish)

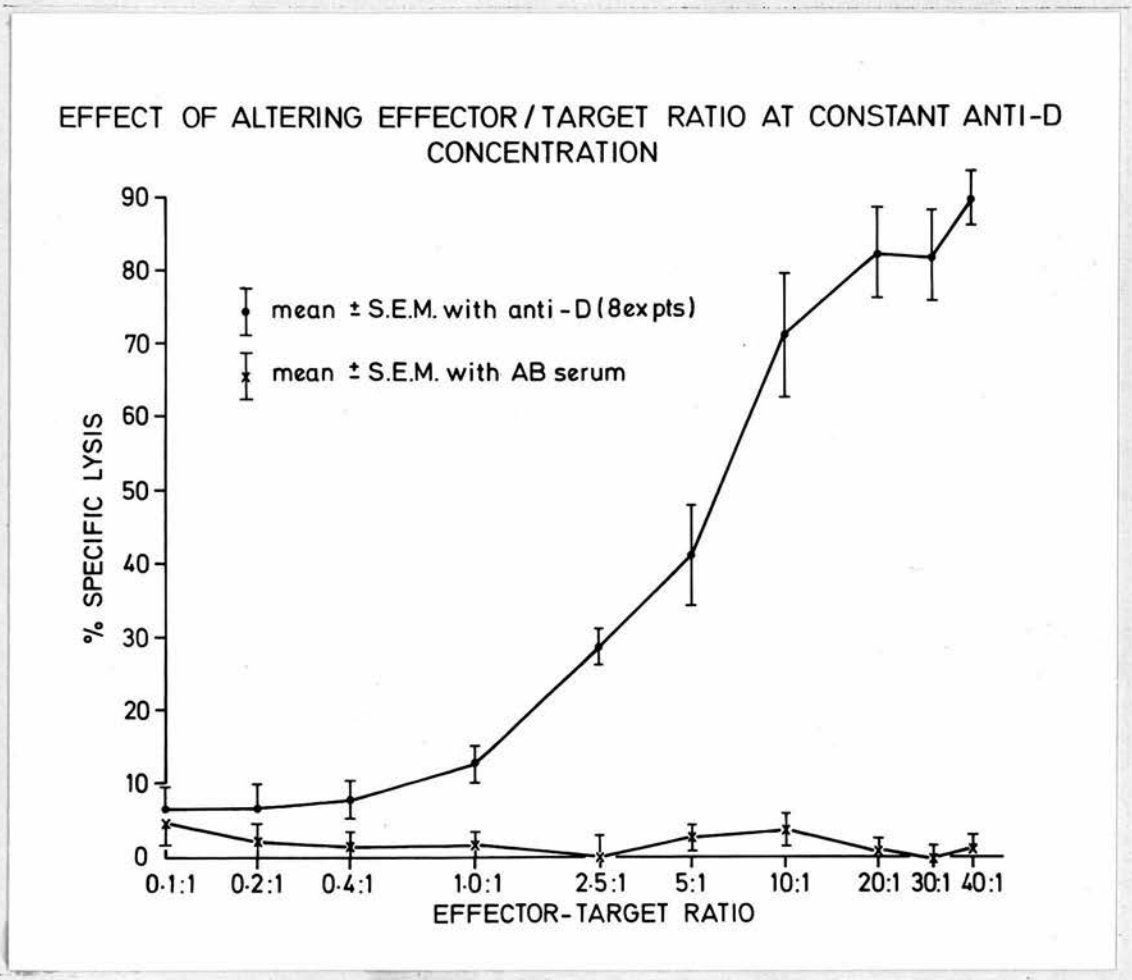

Fig. 5.1 (data from table 5.1)

5.2 Removal of monocytes by nylon wool columns

When the technique of nylon wool removal of monocytes was introduced, it was felt important to repeat the above experiments to see if the more efficient removal of monocytes resulted in a significant loss of specific lysis, since a number of workers have reported that the monocyte is largely responsible for K-cell lysis of red cell targets (Holm 1972; Holm & Hammarström 1973; Holm et al 1974).

The results of a series of experiments where the effects of nylon columns were assessed are shown in table 5.2 and the mean values plotted in fig. 5.2.1. A sigmoid curve is again obtained, with a relatively linear increase in % S.L. from 0.2:1 (1:5) to 5:1. Below 0.1:1 specific lysis is not significantly above background (except in a few individuals) and above 5:1 a plateau effect is observed at approximately 70% S.L.

It is informative to compare these results with those shown in fig. 5.1. On comparing the two groups of experiments on the basis of the E/T ratio there is apparently much greater lytic activity at lower ratios in the post nylon experiments. However, when the results are expressed in terms of the numbers of effector cells in culture at the various ratios then the curves obtained can be more or less superimposed and the linear part of the sigmoid curves obtained are essentially similar (fig. 5.2.2). Furthermore, the 50% lysis point occurs where a similar number of effector cells are present in culture ie. at approximately 6×10^4 per culture (fig. 5.2.2). This tends to suggest that it is the number of

Table 5.2 EFFECT OF ALTERING EFFECTOR/TARGET CELL RATIO

PRE AND POST NYLON WOOL COLUMN PASSAGE

Lym. donor group	anti-D	*** % monos	Effector - target ratio					
			1/10	1/5	1/1	5/1	10/1	20/1
			0.4*	0.8	4	20	40	80
1. AB R_1r	Louden 1/6 free	15	32.5**	-	45.2	68.9	80.4	84.2
2. A R_1R_1	IgG III 1/3 free	22.5	3.5	-	12.8	-	47.5	33.3
3. O R_1R_1	Louden 1/3 free	14.5	14.2	18.5	51.4	91.0	100	97.9
4. O R_1R_1	Louden 1/3 free	10.0	8.8	-	53.7	-	71.8	-
5. O R_1r	Louden 1/3 free	12.5	7.0	11.7	16.5	26.3	28.4	25.7
	Mean	14.9	13.2	15.1	35.9	62.1	65.6	60.3
	S.D.	4.7	11.5	4.8	19.7	32.9	28.1	36.1
	SEM	2.1	5.1	3.4	8.8	19	12.6	18.1
1a as above	as above	0	0.9	6.0	55.5	61.0	98.0	88.9
2a		5.5	8.7	-	22.9	-	32.4	29.5
3a		2.0	19.7	29.3	63.2	87.4	84.1	93.1
4a		1.7	19.5	-	59.3	-	68.5	-
5a		0.7	8.9	17.1	19.5	52.1	54.9	66.7
	Mean	1.9	11.5	17.5	44.1	66.8	67.6	69.5
	S.D.	2.1	8.0	11.6	21.1	18.4	25.5	29.1
	SEM	0.9	3.6	6.7	9.4	10.6	11.4	14.6

* x 10^4 effectors per culture (4×10^4 O R_1R_1 RBC per culture) 1-5 pre nylon; 1a-5a post nylon (same donors).

** % specific lysis. 18 hr. cultures : final vol. 150 µl : anti-D free in culture at dilution shown

*** estimated by phase contrast

effector cells that determines the degree of specific lysis.

At the E/T ratio of 10:1 for the post nylon series (40×10^4 effector cells in culture) the mean percent S.L. was 67.6% and this is not a significant difference. It is likely however that this is coincidence since the actual numbers of RBC lysed in the post nylon series is four times greater. This is also the case for the 50% lysis point, and to be truly comparable 50% lysis should therefore be standardised for the number of available RBC.

A reduction in monocytes from a mean of 14.9% to 1.9% in this particular series of experiments has not resulted in loss of K-cell activity (in fact the opposite - fig. 5.2.1) and there does not appear to be any correlation between the numbers of monocytes in culture and the % specific lysis. The pre and post-removal populations produced similar dose-response curves as the E/T ratio is altered (fig. 5.2.1) with an increase in % specific lysis post-nylon, but these differences are not statistically significant (paired t test).

Pre-nylon cultures contain on average 14.9% monocytes and at an E/T ratio of 1:1 produced 35.9% specific lysis whereas following nylon adherence 1.9% monocytes per culture in presence of the same number of target cells produced a mean of 44.1% specific lysis. If specific lysis were due to monocytes alone then in the first instance one monocyte would lyse approximately 2 RBC per culture whereas in the second instance, under identical

culture conditions, one monocyte will apparently lyse 22 RBC. It is highly unlikely that there are minor differences between the two sets of culture conditions (which were performed on the same day with the same reagents) which would produce a 10-fold difference in the lytic ability of the monocytes. The same argument applies at the other E/T ratios. One therefore draws the conclusion that cells other than monocytes are active in ADCC lysis of RBC. (investigated further in section III).

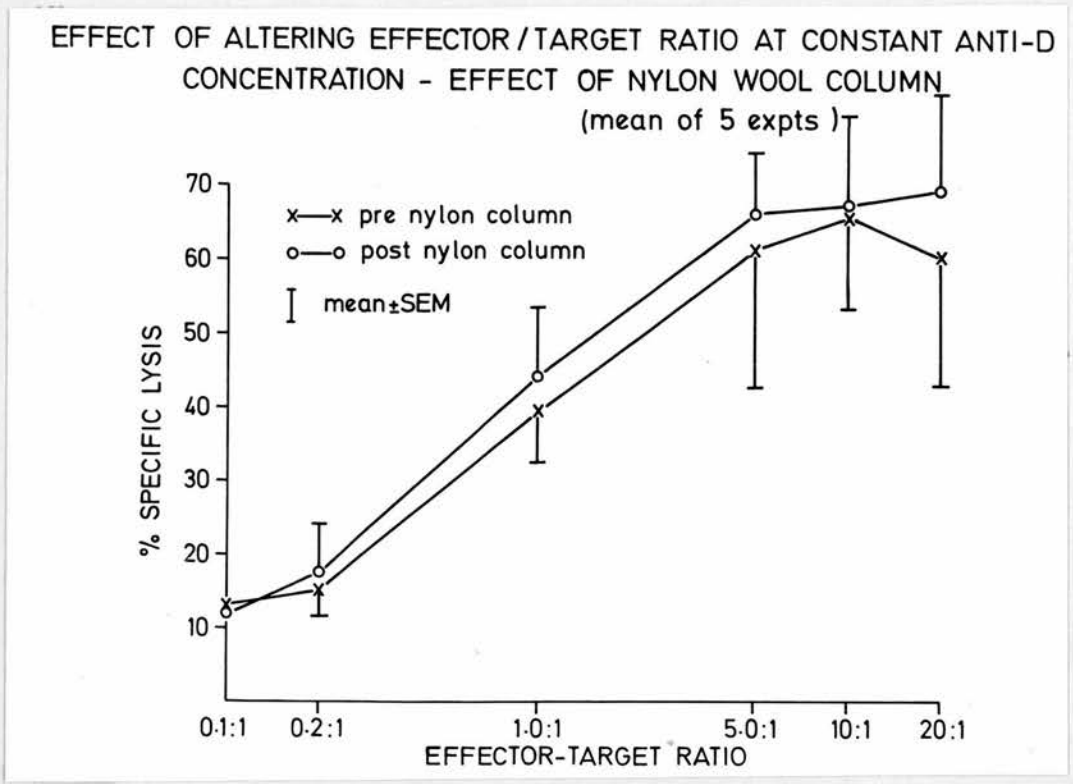

Fig. 5.2.1

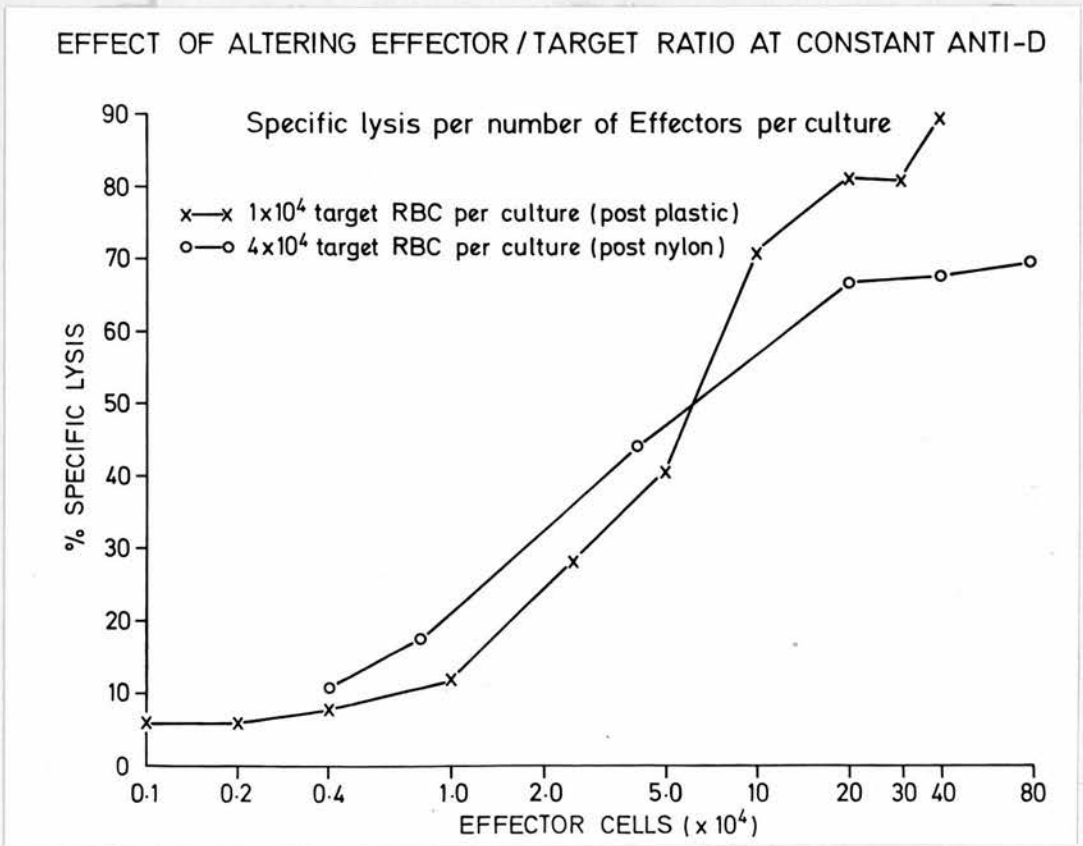

Fig. 5.2.2

5.3 Alteration of E/T ratios at several anti-D concentrations

It has already been noted that provided there is excess antibody present, the efficiency of specific lysis depends on the available number of effector cells. At a given E/T the % specific lysis should depend on the availability of sensitised antibody and this is shown in fig. 5.3 where three anti-D dilutions (1/16; 1/24 and 1/96) have been compared at various E/T ratios with cells from the same donors. A series of approximately parallel lines was obtained corresponding to the linear part of the E/T dose-response curve (see fig. 5.2.2) and the % S.L. at a given E/T fell as the anti-D serum was diluted. However the reduction in % S.L. with antibody dilution is not linear in that a four-fold dilution of anti-D from 1/6 to 1/24 at E/T of 10:1 reduced the % S.L. by only a factor of 1.4 and similar calculations can be made at other E/T ratios. It is likely therefore that a relatively small amount of antibody is effective in inducing K-cell lysis and this is investigated further in para. 6.0.

5.4 Conclusions

When the E/T ratio is altered at a given fixed concentration of anti-D (calculated to be an excess), a sigmoidal dose-response type of curve obtained, varying from no observed lysis at low E/T to a plateau in specific lysis at high E/T ratios. The same shape of curve is obtained if monocytes are depleted by either plastic or

or nylon adherence and the % specific lysis appears independent of the numbers of monocytes per culture. Effector cells from different individuals can be compared by estimating the 50% specific lysis point but this gives only a functional and not numerical estimate of K-cell activity.

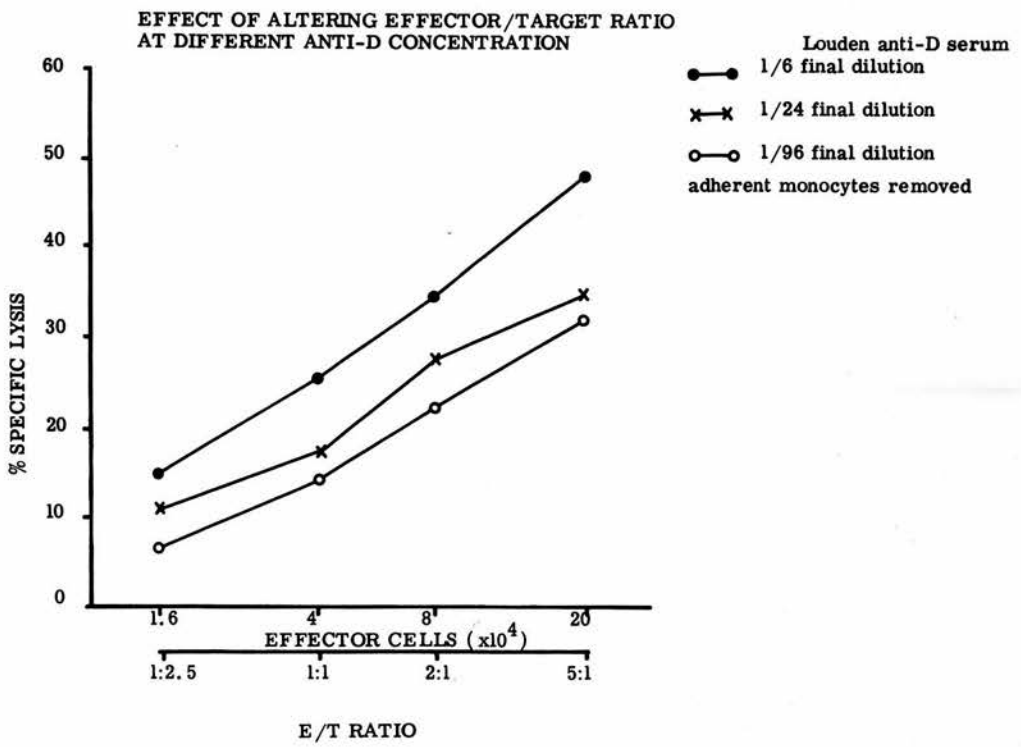
EFFECT OF ALTERING EFFECTOR/TARGET RATIO
AT DIFFERENT ANTI-D CONCENTRATION
Louden anti-D serum
1/6 final dilution
1/24 final dilution
1/96 final dilution
adherent monocytes removed
% SPECIFIC LYSIS
60
50
40
30
20
10
0
1.6
4
8
20
EFFECTOR CELLS (x10^4)
1:2.5
1:1
2:1
5:1
E/T RATIO

Fig. 5.3

6.0 ALTERATION OF ANTI-D CONCENTRATION AT FIXED EFFECTOR/TARGET CELL RATIO

It has been suggested previously (para. 5.0) that anti-D was present in excess and that changes in % specific lysis reflect only the availability of effector cells in culture. A series of experiments were therefore carried out to demonstrate the antibody requirements more formally. The following results demonstrate the effect of diluting IgG anti-D and serum anti-D and the effect of removing monocytes.

6.1 Dilution of IgG anti-D fraction

Louden IgG anti-D (chapt. III para. 8.1b) was used in ADCC assays standardised at an E/T of 10:1, 18 hr. culture and monocytes removed prior to assay. Two series of experiments are reported, one with monocyte removal on plastic dishes (table 6.1a) and the second with nylon column removal (6.1b) and the results are shown in diagramatic form in fig. 6.1.1. A dose-response type of curve is obtained, with a plateau effect up to dilutions of 1/480 and a gradual decrease of % S.L. to barely detectable levels at 1/15,000. The anti-D concentration of this particular anti-D was not accurately determined but was in the region of 240 µg/ml, in the undiluted fraction, and a dilution of 1/15,000 therefore indicates activity at approximately 0.02 µg/ml. An average maximum of 60% S.L. is seen at the plateau. There is a suggestion of a prozone effect at the lower dilutions of 1/30 and 1/60, with maximum % S.L. seen at 1/120. This may be a true prozone effect with inhibition of K-cell

lysis due to saturation of both the RBC target antigens and the effector cell Fc receptor by an excess of anti-D molecules. A similar effect has been reported by Zeijlemaker et al (1975) using a transformed cell-line target. It is also possible that there is "non-specific" blocking of the Fc receptors by "irrelevant" IgG molecules similar to the inhibition of Fc-rosette binding by monocytes in the presence of high concentrations of serum (containing IgG) (Abramson et al 1970a). This is less likely however since maximal K-cell lysis is observed in a minimum of 10% human serum, and more usually, in the region of 40% human serum (100 µl at 10% and 50 µl at 100% in cultures where 50 µl neat serum is added to give final volume of 150 µl). This contrasts with the inhibition of monocyte-mediated RBC lysis at low serum dilutions reported by Holm et al (1974). The two monocyte removal processes have been analysed separately and shown in tables 6.1a and 6.1b and in fig. 6.1.2. The culture conditions are in all cases identical apart from the different removal procedures and donors. It can be seen that with the more efficient removal of monocytes with nylon wool (mean 2.4% range 0-4%) there is in fact an overall increase in percent specific lysis but the differences are not statistically significant (paired t test). This evidence suggests that monocytes are not active in this system. The individual variation of K-cell activity in the presence of equivalent concentrations of anti-D and numbers of effector and target cells was quite marked and the responses of three individuals are shown in fig. 6.1.3.

Table 6.1a ALTERATION OF ANTI-D

CONCENTRATION AT FIXED E/T RATIO

RBC donor	Lym. donor	Anti-D dilution											
		1/30	1/60	1/120	1/240	1/480	1/960	1/1800	1/3000	1/6000	1/9000	1/12000	1/15000
1. O R_1R_2	O R_1R_2	61.3	-	61.0	40.1	34.0	38.2	-4.7	-7.3	-	-	-	-
2. O R_1R_1	O R_1r	35.3	40.4	34.9	28.6	29.9	20	32.2	19	-	-	-	-
3. O R_1R_1	O R_1R_2	59.3	55.7	44.1	37.3	28.9	24.1	15.1	11.1	-	-	-	-
4. O R_1R_1	O R_1R_1	75.6	83.5	89.0	71.9	74.9	68.5	54.8	44.1	-	-	-	-
5. O R_1R_1	AB R_1R_1	45.6	38.3	44.4	47.7	37.2	41.3	47.8	38.8	-	-	-	-
6. O R_1R_1	AB R_1R_1	-	78.9	102.3	78.9	73.5	67.2	62.9	86.1	-	-	-	-
7. O R_1R_1	A R_1R_2	-	-	87.7	63.1	86.3	49.2	62.4	32.8	-	-	-	-
	mean for 1-7	55.4	59.4	66.2	52.5	52.1	44.1	38.6	32.1	-	-	-	-
	S.D.	15.5	21.1	26.6	19.0	24.9	19.0	25.7	25.6	-	-	-	-
	S.E.M.	6.9	9.4	10.1	7.2	9.4	7.2	9.7	11.2	-	-	-	-

effector/target ratio 10:1 (1×10^4 RBC per culture) 18 hr. cultures with anti-D IgG I concentration as shown

1 - 7 : plastic dish removal of monocytes

- = not tested

Table 6.1b ALTERATION OF ANTI-D

CONCENTRATION AT FIXED E/T RATIO

RBC donor	Lym. donor	Anti-D dilution 1/30	1/60	1/120	1/240	1/480	1/960	1/1800	1/3000	1/6000	1/9000	1/12000	1/15000
8. O R_1R_1	AB R_1R_1	-	-	81.5	79.9	76.6	70.5	61.9	-	19.3	15.1	13.1	7.1
9. O R_1R_1	A R_1r	-	-	81.3	59.1	58.5	9.9	1.2	22.4	-10.1	2.2	-1.9	-4.4
10. O R_1R_1	O R_1r	-	-	43.1	19.6	32.6	13.2	6.3	4.9	-0.7	1.1	0.2	0
11. O R_1R_2	AB R_1R_1	-	-	69.3	45.5	31.9	29.8	38.3	49.5	29.1	24.4	22.1	9.6
12. O R_1R_2	O R_2r	-	-	89.1	84.9	83.1	89.1	86.1	79.6	67.1	58.2	48.3	32.4
13. O R_1R_2	B R_2r	-	-	72.8	69.9	70.0	68.8	61.5	57.3	50.6	-	28.8	23.0
	mean for 8-13	-	-	72.9	59.8	58.8	46.9	42.6	42.7	25.9	20.2	18.4	11.3
	S.D.	-	-	16.2	24.3	22.1	33.5	33.7	29.4	29.6	23.3	18.9	14.0
	S.E.M.	-	-	6.6	9.9	9.0	13.7	13.8	13.7	12.2	10.4	7.7	5.7
	N	-	-	6	6	6	6	6	5	6	5	6	6
	Total mean for 1-13	55.4	59.4	68.3	55.9	55.2	43.9	40.5	36.5	25.9	20.2	18.4	11.3
	S.D.	15.5	21.1	22.5	21.0	22.9	28.3	28.4	28.7	29.6	23.3	18.9	14.0
	S.E.M.	6.9	9.4	6.2	5.8	6.4	7.8	7.9	8.3	12.2	10.4	7.7	5.7

effector/target ratio 10:1 (1 x 10^4 RBC per culture) 18 hr. cultures with anti-D IgG I concentration as shown

8 - 13 : nylon wool removal of monocytes

- = not tested

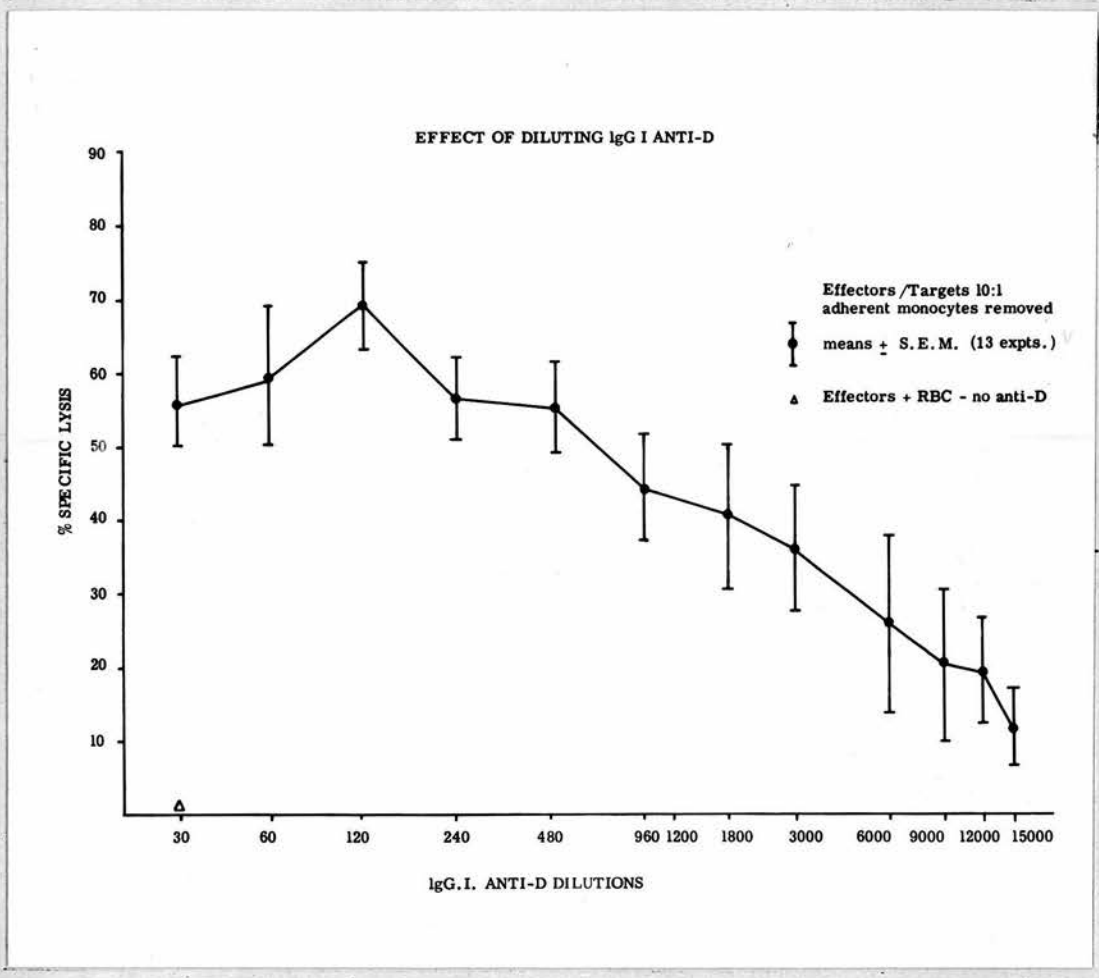

Fig. 6.1.1

(data from tables 6.1a and 6.1b)

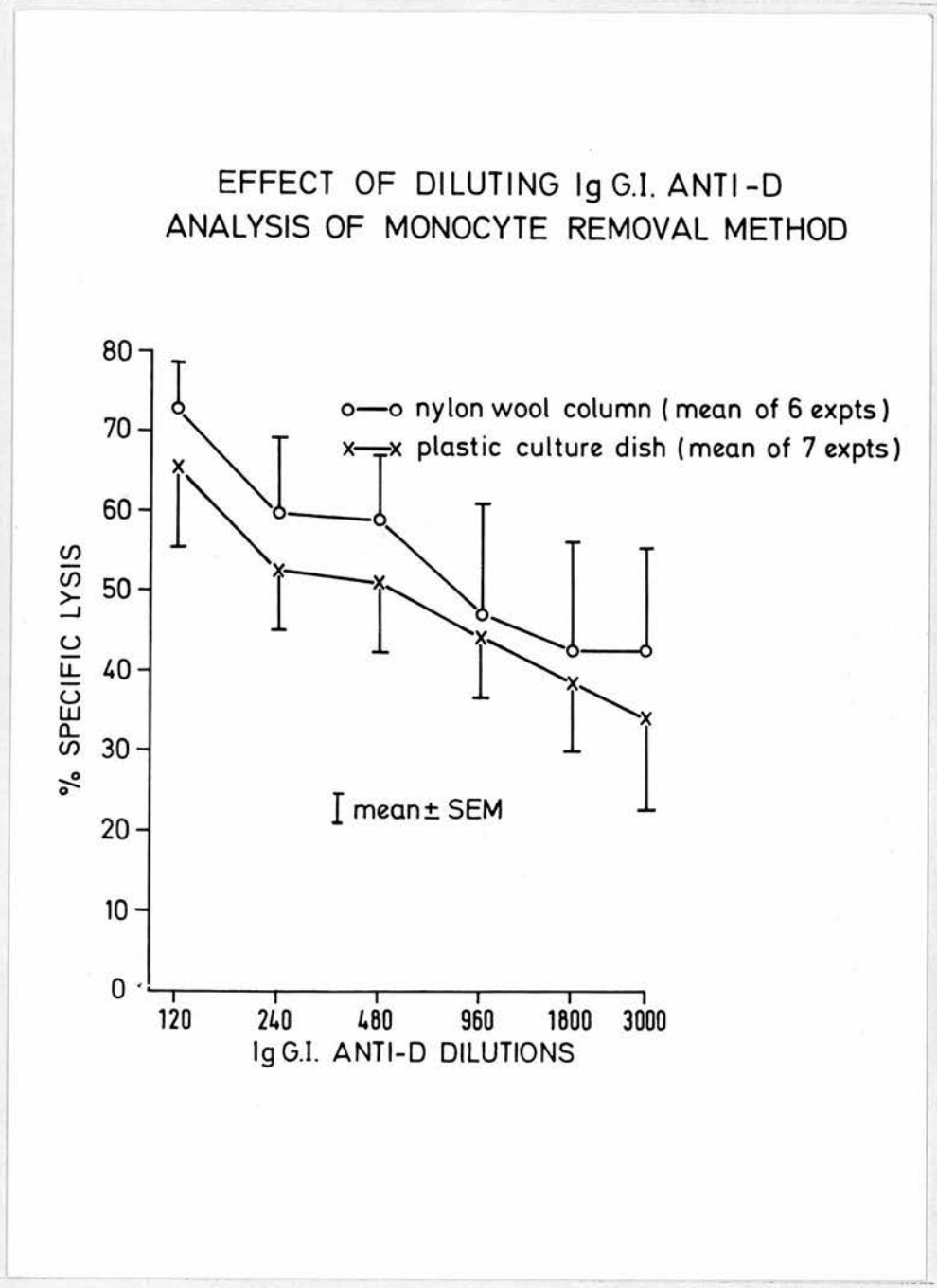

Fig. 6.1.2

(data from tables 6.1a and 6.1b)

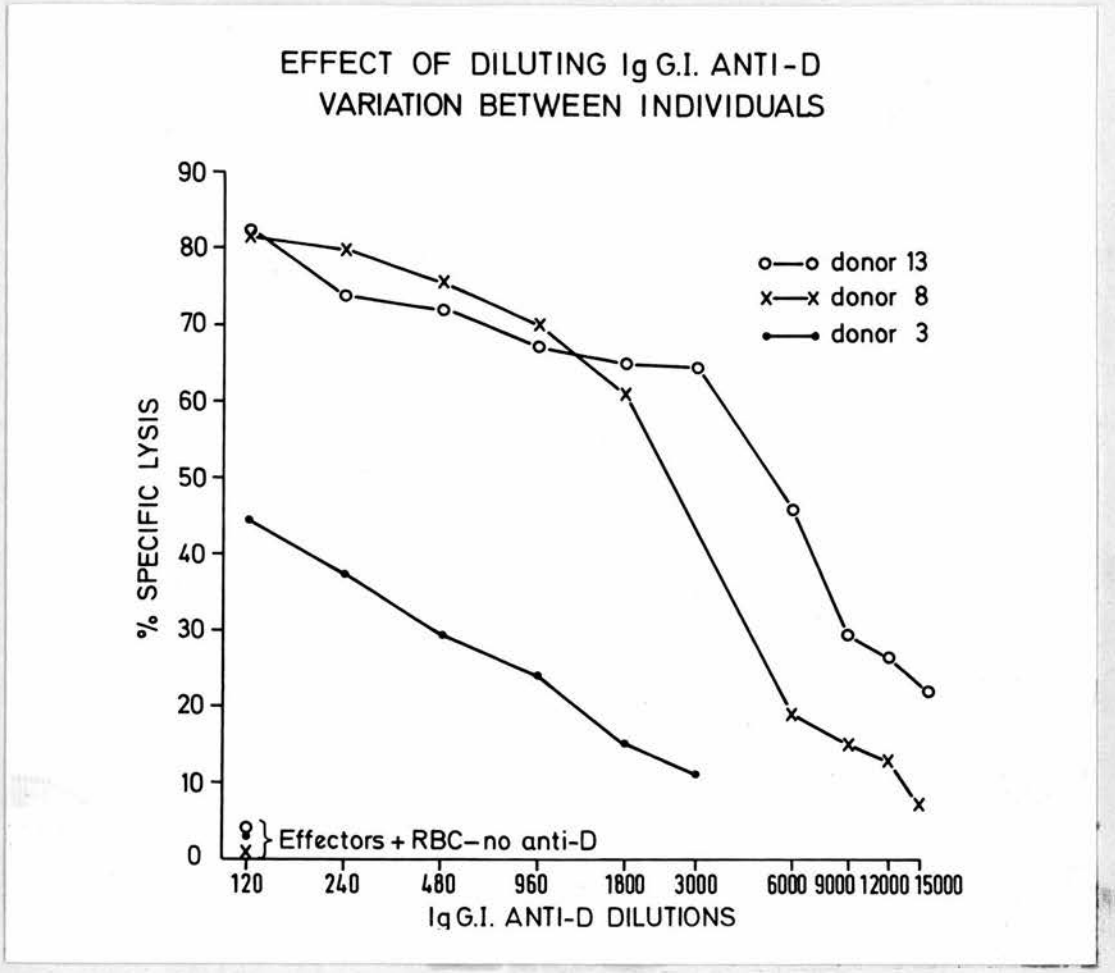
EFFECT OF DILUTING Ig G.I. ANTI-D
VARIATION BETWEEN INDIVIDUALS
% SPECIFIC LYSIS
90
80
70
60
50
40
30
20
10
0
o—o donor 13
x—x donor 8
•—• donor 3
Effectors + RBC—no anti-D
120
240
480
960
1800
3000
6000
9000
12000
15000
Ig G.I. ANTI-D DILUTIONS

Fig. 6.1.3

All three donors show a "dose response" curve but the shape of the curve varies. This probably reflects differences in K-cell lytic capacity rather than the availability of anti-D which is the same for each donor. It is pertinent to note that the end-point of the antiglobulin test for this particular anti-D fraction was 1/2048 whereas K-cell activity is observed at much higher dilutions (fig. 6.1.1) indicating the great sensitivity of the K-cell assay under the appropriate conditions.

6.2 Effect of removal of monocytes

It has been shown above that there was little difference in the specific lysis of sensitised RBC with either of the monocyte removal procedures. A few experiments were carried out with anti-D dilutions where K-cell activity was tested both pre and post-removal of adherent cells, by both methods. Representative results are shown in figs. 6.2.1 and 6.2.2. It can be seen that the pre and post monocyte removal cultures behave in a similar dose-response manner in each case, and that there is an increase in % specific lysis after removal of monocytic cells indicating the lack of correlation of ADCC with the presence of monocytes. This was not always observed, but the converse ie. loss of activity was rarely seen.

6.3 Dilution of anti-D serum

A similar dose-response curve was obtained on diluting the original anti-D serum as with the concentrated IgG fraction, and the results of a representative experiment are shown in fig. 6.3.

There is a suggestion of a prozone effect, but this was seldom observed with most donors and maximum % S.L. was obtained at 1/3 to 1/12 dilutions. The end-point of observed lysis is seen at 1/192 and this corresponds to an anti-D concentration of 0.02 μg/ml which is in close agreement with the end-point of lysis noted in fig. 6.1.1 with the same original source of anti-D. This suggests that at this point there are insufficient anti-D molecules per RBC to induce K-cell lysis.

It is interesting to note that the antiglobulin titre of the anti-D serum was only 1/32 whereas it could be diluted up to 1/192 in K-cell lysis again demonstrating the sensitivity of the assay.

6.4 Conclusions

Dilution of anti-D-containing material in the presence of a fixed number of effector and target cells results in a dose-response type of curve. A plateau of maximum lysis is obtained at the highest concentration where anti-D is present in excess followed by a log-linear reduction in % specific lysis on further dilution, indicating that availability of antibody is rate-limiting under these culture conditions. With the anti-D used specific lysis was seen down to 0.02 μg/ml in culture (= 3ng anti-D per 150 μl culture) and the assay demonstrates the presence of appropriate antibody with greater sensitivity than the antiglobulin test.

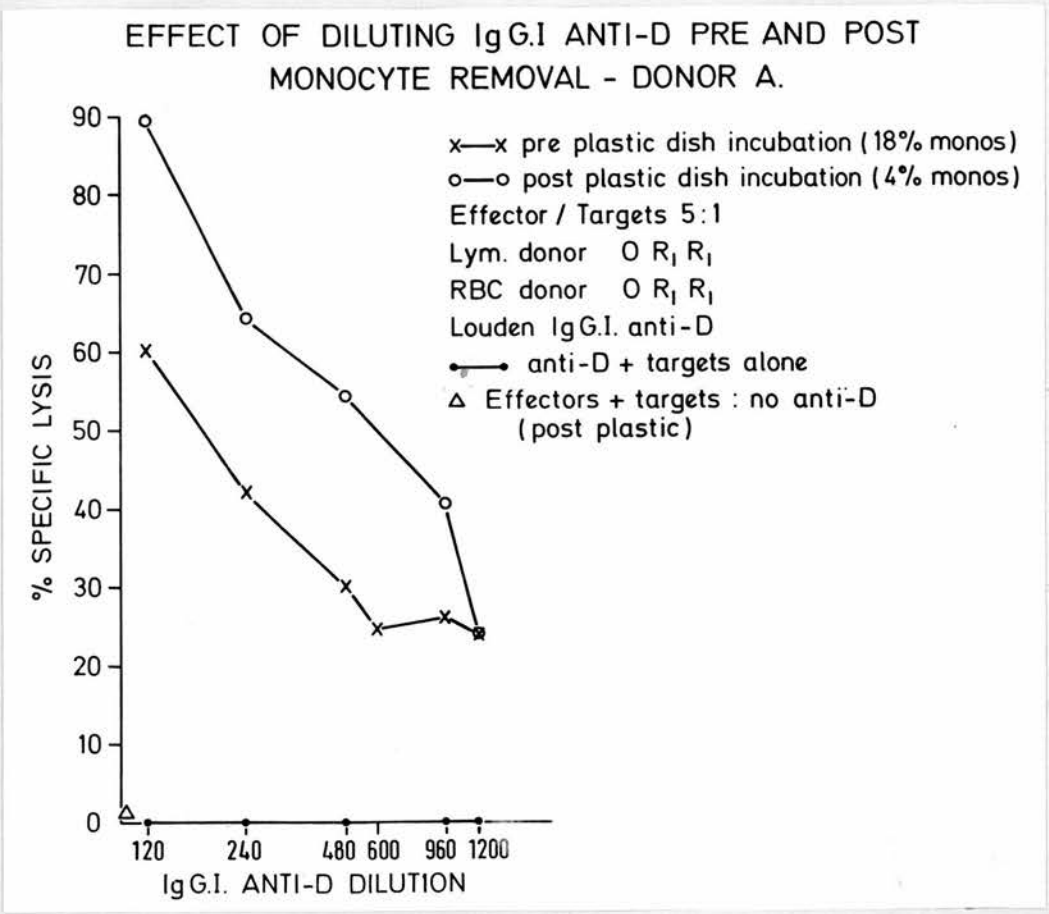

Fig. 6.2.1

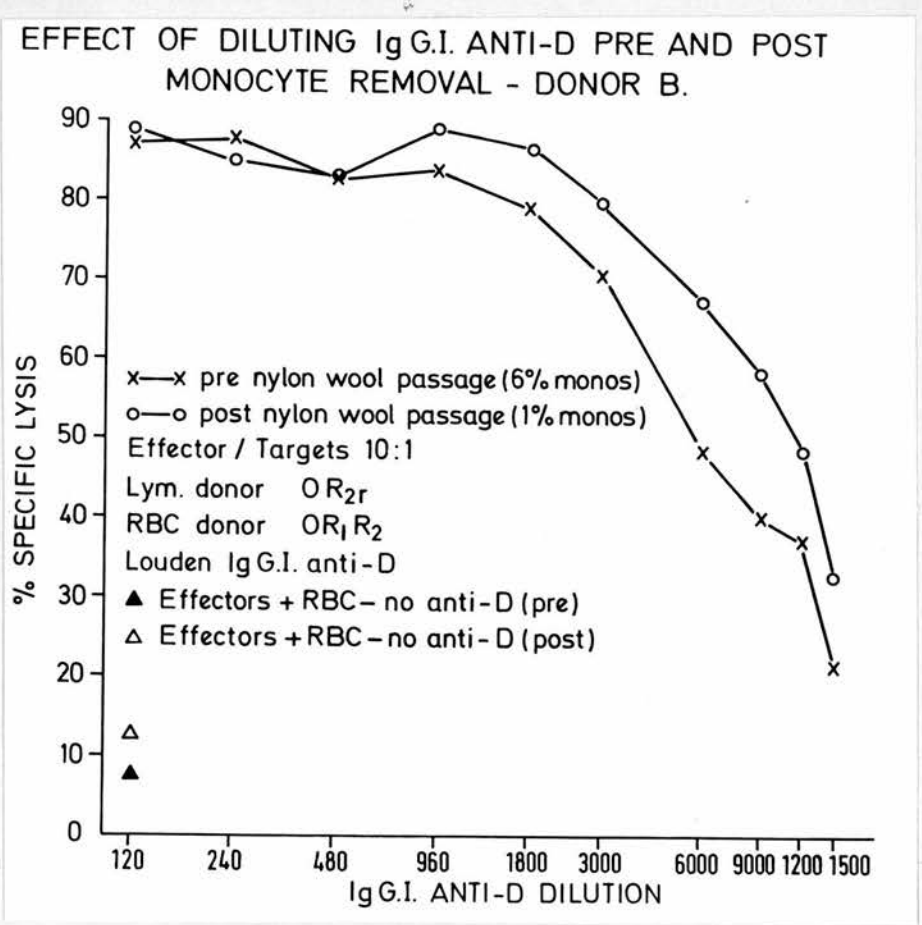

Fig. 6.2.2

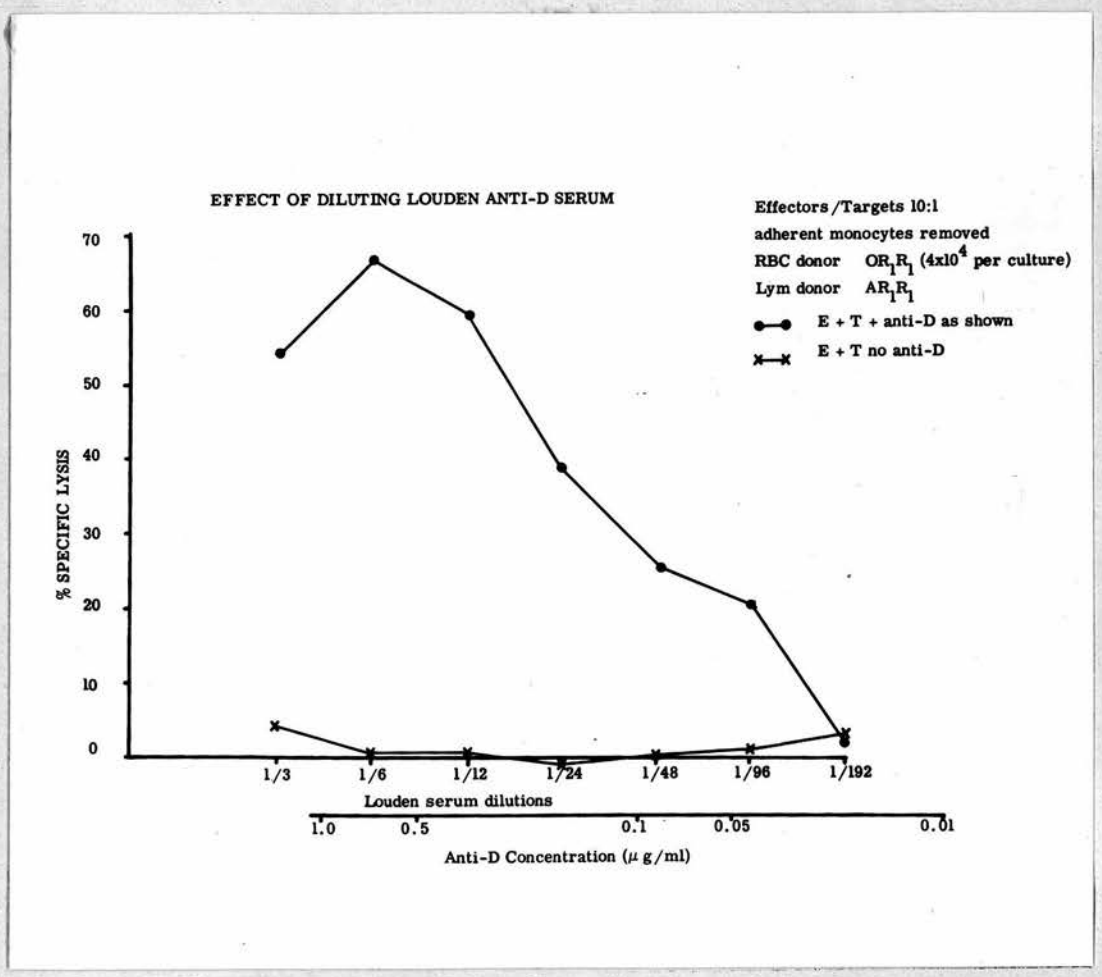
EFFECT OF DILUTING LOUDEN ANTI-D SERUM
Effectors/Targets 10:1
adherent monocytes removed
RBC donor OR_1R_1 ($4x10^4$ per culture)
Lym donor AR_1R_1
E + T + anti-D as shown
E + T no anti-D
% SPECIFIC LYSIS
70
60
50
40
30
20
10
0
1/3
1/6
1/12
1/24
1/48
1/96
1/192
Louden serum dilutions
1.0
0.5
0.1
0.05
0.01
Anti-D Concentration (μg/ml)

Fig. 6.3

7.0 FREE ANTI-D IN CULTURE COMPARED WITH PRE-SENSITISATION OF THE RBC TARGET

The effect of using the same source of anti-D to either pre-sensitise RBC with transfer of cells directly into the cultures, or the direct addition of anti-D-containing material to the cultures, were studied in replicate cultures processed simultaneously.

The results of such comparisons drawn from several experiments over a period of time are shown in table 7.0. It can be seen that with one exception (experiment 2), the % specific lysis is greater with free anti-D in culture than pre-sensitisation of the RBC, although in the final cultures there are identical numbers of effector and target cells.

Comparing the means of the two groups, the differences between presensitised target RBC and free anti-D in culture are statistically significant (paired t test : $p < 0.005 > 0.0025$). This is not surprising considering the relative availability of antibody in the two systems.

With presensitisation of RBC by anti-D the majority of D-antigen sites are likely to be occupied by high affinity anti-D and it was confirmed that anti-D was bound to these RBC by the direct antiglobulin technique. However, quantitative determinations were not possible so that the actual amount of anti-D bound per RBC could not be assessed.

Where an amount of anti-D was added directly to the

Table 7.0 EFFECT OF FREE ANTI-D IN CULTURE AND PRESENSITISATION OF RBC TARGETS K-CELL LYSIS

	E/T* ratio	Lym. donor group	RBC donor group	anti-D** source	% specific lysis with	
					presensitised	free anti-D
1.	5:1	AB R_1R_1	O R_1R_1	Louden N : 1/3	22.0	44.6
2.	10:1	AB R_1R_1	O R_1R_2	Cumming 1/10 : 1/60	70.0	64.7
3.	10:1	O R_1r	O R_1r	IgG I N : 1/6	32.0	82.0
4.	10:1	-	O R_1R_2	Cumming 1/10 : 1/60	52.5	54.5
5.	10:1	O R_1r	O R_1R_1	Louden N : 1/3	10.3	57.2
6.	10:1	B rr	O R_1R_1	IgG II 1/20 : 1/60	32.7	57.7
7.	10:1	B R_1R_1	O R_1R_1	IgG II 1/20 : 1/60	34.9	55.9
8.	10:1	O R_1r	O R_1R_1	Louden N : 1/3	59.9	74.2
9.	10:1	O R_1R_2	O R_1R_1	Louden N : 1/3	19.0	32.1
				mean	37.03	58.1
				S.D.	19.9	14.8

* Effector/target ratio: 1-3 = 1×10^5 RBC ; 4-9 = 0.4×10^5 RBC

** Final dilution to presensitise 20×10^6 RBC (N = neat undiluted), or free in culture

Difference between means is statistically significant ($p < .005 > 0.0025$) (paired t test)

cultures the antibody is present throughout the time of culture. Binding to D-antigen sites should be greater and there will not be any loss of anti-D following washing as with the preparation of the pre-sensitised RBC. There are also less RBC in these final cultures ($0.4 - 0.1 \times 10^5$ RBC compared with 200×10^5 RBC) and there will be a greater excess of anti-D per RBC.

In view of the intention to use the K-cell assay to determine RBC D antigen zygosity, and the requirement for pre-sensitised RBC in inhibition assays, the above culture conditions were investigated in more detail as shown below.

7.1 Alteration of E/T ratio

The effect of altering the E/T ratio with free anti-D and pre-sensitised target cells is shown in fig. 7.1.1 and 7.1.2. It is interesting to note the differences with alternative anti-D sources. In fig. 7.1.1 it can be seen that there is considerable lysis even at 2.5:1 with both free anti-D (47.4% S.L.) and with pre-sensitised RBC (43.1% S.L.). The degree of lysis shown with pre-sensitised RBC in this particular instance contrasts with the results shown in fig. 7.1.2 where a different anti-D was used (experiment 5, table 7.0). Here the pre-sensitised RBC target exhibits very little lysis even at an E/T ratio where there is very high percent S.L. with anti-D in culture. The differences between these two experiments suggest that the affinity of anti-D-binding to the RBC may be important for the

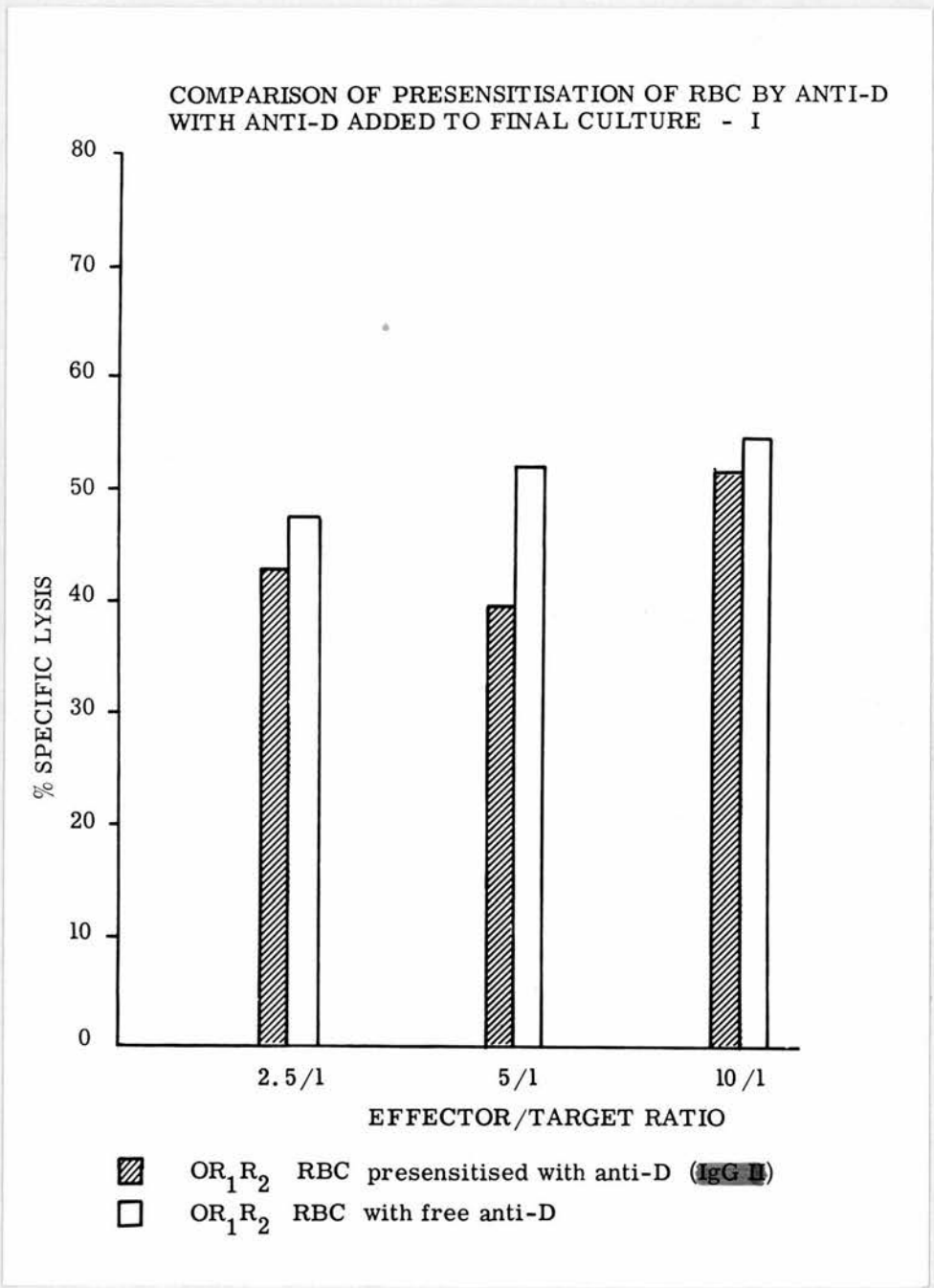

Fig. 7.1.1

(donor 2, table 7.0)

Note: Cumming anti-D serum

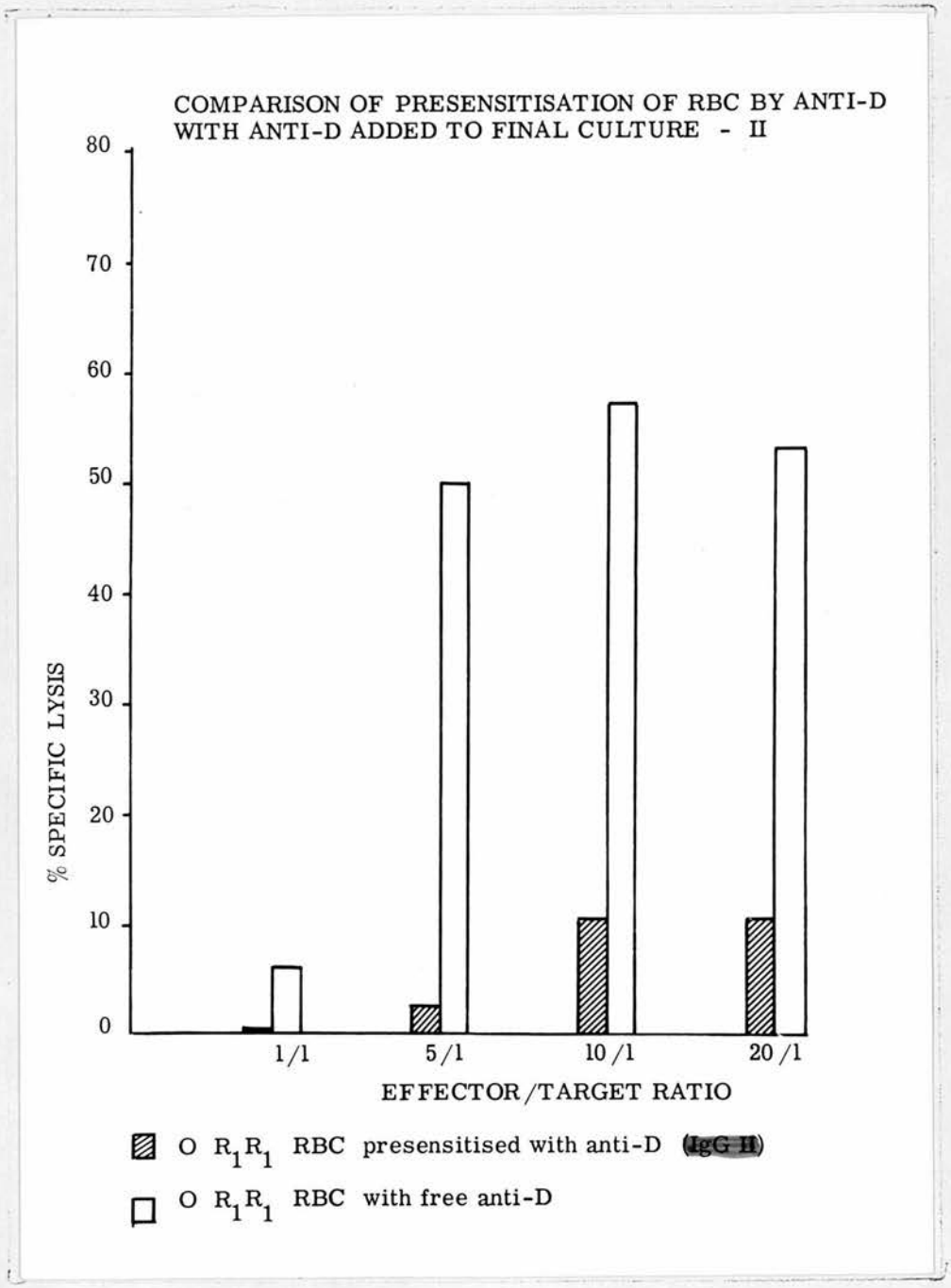

Fig. 7.1.2

(donor 5, table 7.0)

Note: Louden anti-D serum

pre-sensitised RBC to show a high % specific lysis since less anti-D will be lost during the washing procedures. Unfortunately, since a quantitative assay was unavailable this could not be confirmed.

7.2 Alteration of incubation time

The enhanced effect of lysis in the presence of free anti-D is seen only at the more prolonged incubation times (>2-4 hours culture) as shown in figs. 7.2.1, 7.2.2 (donors 6 & 7, table 7.0). The lack of significant difference at the shorter incubation times is possibly because the cultures containing pre-sensitised RBC are initially susceptible to rapid lysis since they already have cell bound anti-D, whereas in the experiments where anti-D is added to the cultures a certain amount of time has to lapse before sufficient amounts of anti-D become bound to the RBC to activate K-cell lysis. The pre-sensitised anti-D coated RBC will also spontaneously aggregate on contact in culture and may settle more quickly than the unsensitised red cells and thus allow contact with the K-cells a little sooner than with the unsensitised red cells. However, over the longer incubation period, the effect of having higher available amounts of anti-D in culture results in a higher final % specific lysis.

7.3 Conclusions

With effector and target cells prepared from the same donors greater % specific lysis is seen with anti-D present during the incubation period than if the RBC are pre-sensitised in an excess of antibody. This probably

relates to the greater availability of anti-D in culture since there is an excess under the condi tions studied. Apart from the difference in end-point specific lysis, the characteristics of the K-cell assay are very similar and either free or RBC bound anti-D can be used for detailed investigation of the mechanism of lysis.

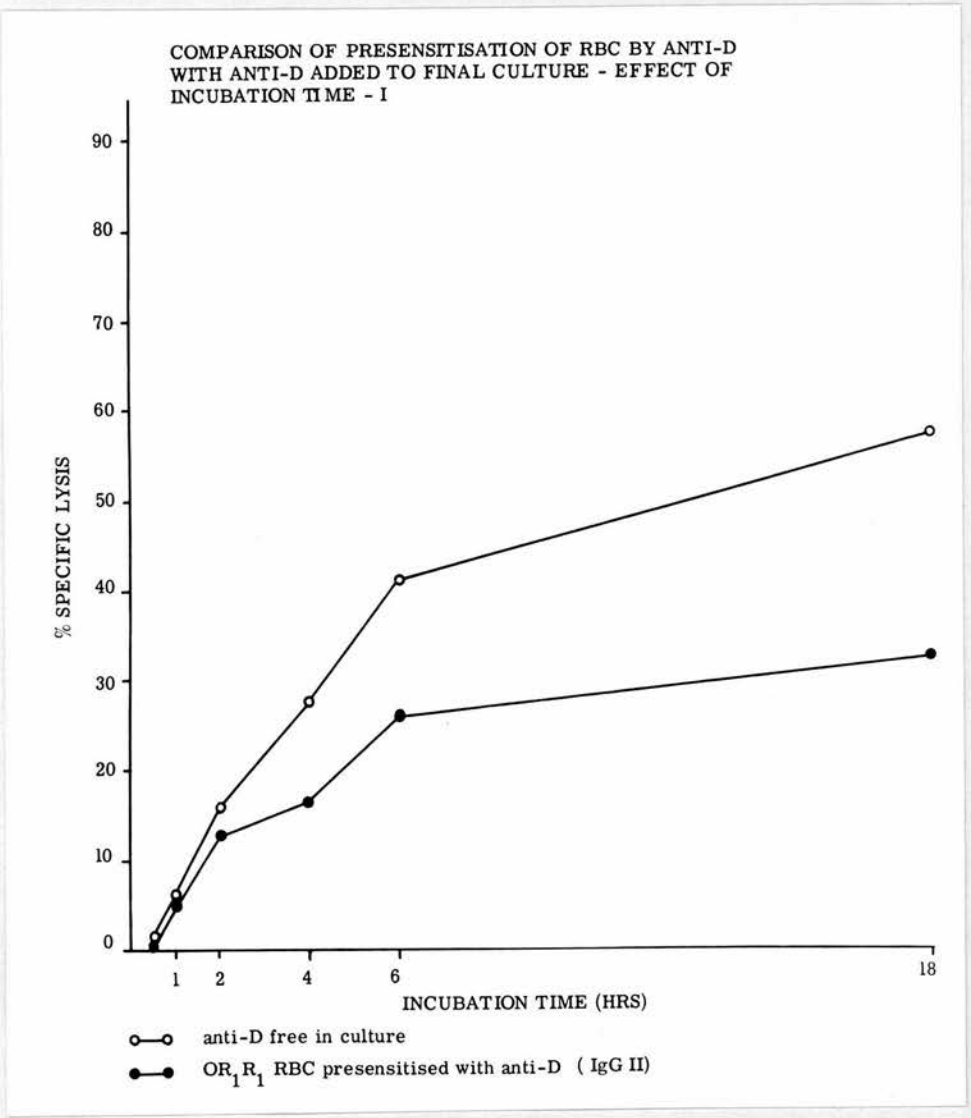

Fig. 7.2.1

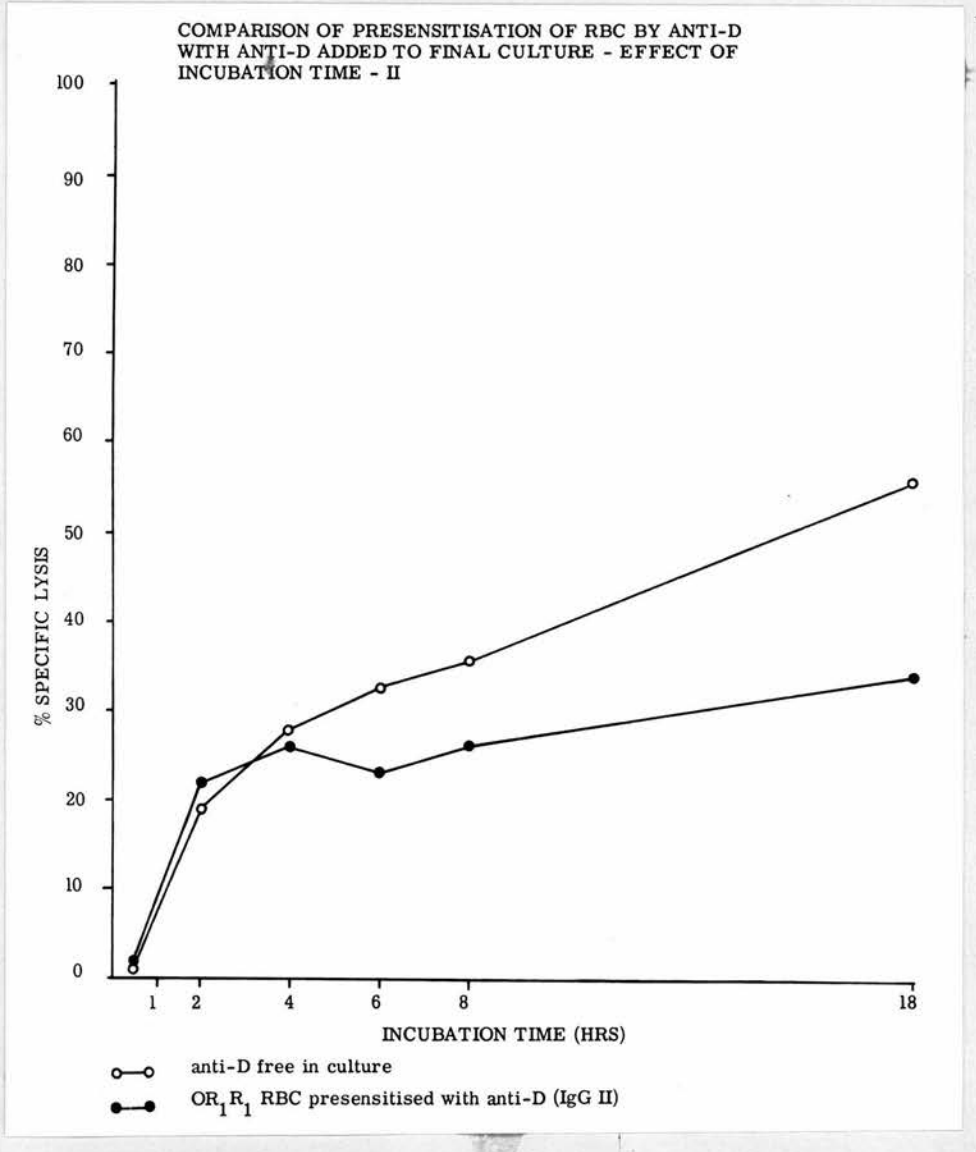

Fig. 7.2.2

8.0 SPECIFICITY OF ANTIBODY FOR K-CELL LYSIS

K-cell donors of various blood group types are equally able to lyse D positive RBC in the presence of anti-D (many of previous tables) and are also able to lyse autologous red cells in the presence of anti-D (donor 1, table 3.1, donor 3, table 5.1).

Whilst it was initially assumed that specificity for lysis resided with the antibody and not the mononuclear cells (as reported in the literature for other systems), it was thought necessary to investigate this more formally.

To confirm the antibody specifity against D-positive RBC controls were set up with anti-D and O rr RBC (ie. negative for the putative antibody specificity). As shown in table 8.0, O rr RBC are not lysed in presence of anti-D and effector cells, and have a similar ^{51}Cr release whether in anti-D or antibody-free group AB serum; the degree of specific lysis is similar to that seen with effector cells and D positive RBC in the presence of antibody-free AB serum i.e. only background release.

This indicates that the appropriate combination of antigen-antibody is required for lysis by K-cells. The same pattern of reactions is seen with anti-D free in the culture medium or with pre-sensitised RBC ie. where only cell bound antibody is transferred with the sensitised red cells into the final culture mixture.

8.1 Alteration of E/T ratio

The lysis of D positive red cells is not an "all or none" phenomenon but depends on the amount of antibody

Table 8.0 SPECIFICITY OF ANTI-D MEDIATED LYSIS FOR D POSITIVE RBC

	E/T ratio	Lym. donor group	ant-D source	% specific lysis with			
				D pos (O R_1R_1) RBC		D neg (O rr) RBC	
				+ anti-D	+ AB serum*	+ anti-D	+ AB serum*
1	5:1	O rr	IgG I. 1/30 free	90.7	-1.2	-3.3	-3.9
2	10:1	O rr	IgG I. 1/30 free	30.9**	0	-5.6	-3.4
3	10:1	O rr	presens. IgG III	34.0	NT	1.1	NT
4	10:1	A R_1R_1	presens. IgG III	36.4	NT	-1.2	NT

* in culture at same dilution as anti-D source

** O R_1R_2 targets on this occasion

NT = not tested

1 and 2 1 x 10^5 RBC 3 and 4 0.4 x 10^5 O R_1R_1 RBC

All 18 hr. cultures except 4 (5 hr. culture) : adherent monocytes removed

present (para. 6.0) and on the number of effector cells present in culture (para. 5.0). This latter effect is illustrated in fig. 8.1 and tables 8.1.1 and 8.1.2 where three different donors have been studied over a range of ratios in the presence of the same final total amount of anti-D per culture. A "dose response" pattern was obtained in each case and this occurs with both free anti-D in culture or pre-sensitised RBC.

At the lower E/T ratios ie. 1:10 the effector cells are probably the greater limiting factor in the presence of an excess of anti-D and RBC, and there is very little more than background lysis. Individual variation is seen in the degree of lysis obtained at the end of the incubation period which may reflect differences in the K-cell population between individuals (see section IV) but in each case the D-negative RBC are not lysed despite an active K-cell population in culture.

8.2 Alteration of incubation time

The effects of K-cells on D-positive and D-negative RBC were examined at different times in culture and the results are shown in table 8.2. It can be seen again that the D-negative RBC are not lysed in the presence of anti-D whereas the D-positive RBC are effectively lysed and that cytotoxicity increases with the length of incubation time. In this particular experiment the RBC were pre-sensitised with anti-D and the effector cells promoted maximum lysis in a short incubation time and thus the rate-limiting factor is the amount of time

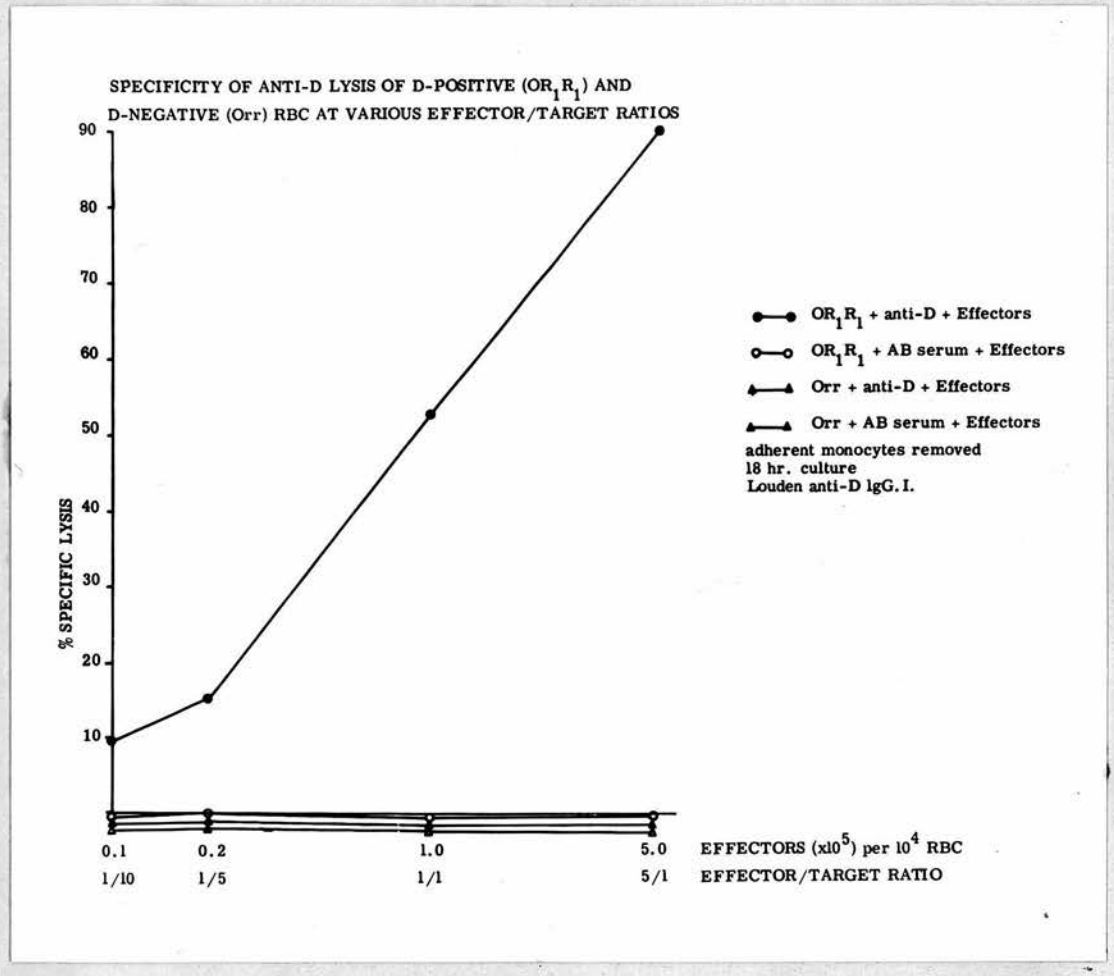

Fig. 8.1

(donor 1, table 8.0)

Table 8.1.1 SPECIFICITY OF ANTI-D LYSIS - EFFECT OF ALTERING EFFECTOR/TARGET RATIOS - I

E/T ratio	% specific lysis with			
	D pos ($O^{r}R_1R_2$) RBC		D neg (O rr) RBC	
	+ anti-D*	+ AB serum	+ anti-D	+ AB serum
10:1	30.9	0	-5.6	-3.4
5:1	21.2	-8.2	-8.9	-1.8
1:1	15.5	-4.0	2.9	-5.0
1:5	10.5	-5.1	-2.5	-0.7
1:10	2.3	0	-6.5	-0.7

* IgG I anti-D at final dilution 1/30

** AB serum final dilution 1/30

1×10^5 RBC per culture ; adherent monocytes removed ; 18 hr culture

donor 2, table 8.0

Table 8.1.2 SPECIFICITY OF ANTI-D LYSIS EFFECT OF ALTERING EFFECTOR/ TARGET RATIOS - II

E/T ratio	% specific lysis with	
	D pos (0 R_1R_1) RBC	D neg (0 rr) RBC
	+ anti-D*	+ anti-D*
10:1	34.0	1.1
5:1	23.2	1.7
1:1	10.2	0.7
1:5	6.6	0.4
1:10	3.2	4.4

* IgG III anti-D presensitised RBC

4×10^4 RBC per culture; adherent monocytes removed

18 hr. culture

donor 3, table 8.0

Table 8.2 SPECIFICITY OF ANTI-D LYSIS - EFFECT OF CULTURE INCUBATION TIME

Incubation time (hrs)	% specific lysis with	
	D pos (O R_1R_1) RBC	D neg (O rr) RBC
	+ anti-D	+ anti-D
0	-3.9	1.2
2.5	30.1	1.8
5.0	36.4	-1.2

E/T 10:1 (4×10^4 RBC)

* presensitised with neat IgG III anti-D; adherent monocytes removed : 18 hr. culture

donor 4, table 8.0

the effector cells have available to react with the sensitised RBC.

8.3 Absorption of antibody by target RBC

It has been assumed previously that the active antibody in promoting lysis of D-positive RBC was anti-D since no other red cell antibody specificity had been detected in this serum by conventional antibody-screening methods. Further confirmation of specificity was sought by attempting to absorb out the antibody activity with RBC of the appropriate type.

The results of absorption of Louden anti-D serum are shown in table 8.3.1, and the IgG fraction prepared from this serum in table 8.3.2. It can be seen that in each case two absorptions with O R_1R_1 RBC are sufficient to abolish K-cell activity towards a target cell of the same type in culture whereas absorption with O rr RBC had no effect on K-cell lysis of the O R_1R_1 target. The anti-D titres of the corresponding supernatants from O R_1R_1 absorption parallel the K-cell activity, with one absorption being almost sufficient to abolish detection. It is interesting that significant K-cell activity was still detectable after one absorption with O R_1R_1 RBC whereas the indirect antiglobulin titres are barely detectable, again indicating the sensitivity of the K-cell assay in detecting small amounts of anti-D.

The indirect antiblobulin titres were reduced from 1/32 to 1/16 after absorption with O rr cells in

table 8.3.2; this was possibly due to dilution during absorption with red cells. Nevertheless, the K-cell lytic activity was reduced only slightly after absorption with O rr, giving 72.5% S.L. before absorption, 62% S.L. after one absorption and 63.5% S.L. after the second absorption.

These findings confirm that K-cell lysis of D-positive (O R_1R_1) RBC is mediated by anti-D.

8.4 Conclusions

It has been shown that active K-cells from several individuals are unable to lyse D-negative RBC in the presence of anti-D whereas in parallel cultures D-positive RBC are effectively lysed under a variety of conditions including comparisons between free and cell-bound anti-D, alteration of E/T ratio, and alteration of incubation time.

This indicates that the appropriate antigen-antibody combination is required for specificity and that any suitable donor will suffice as a source of effector cells.

It has also been shown that RBC with the appropriate antigen (D positive) will absorb out the antibody responsible for inducing lysis (anti-D).

However, in this series of experiments it has not been formally demonstrated that the D-negative RBC are not resistant to K-cell lysis since an antibody specific for these cells was not included as a positive control due to the unavailability of a suitably active anti-c or anti-e; the likelihood of such resistance to lysis is remote.

Table 8.3.1 ABSORPTION OF CYTOLYTIC COMPONENT OF ANTI-D SERUM WITH D-POSITIVE (O R_1R_1) RBC

		Absorption with			
		D pos (R_1R_1)		D neg (rr)	
	unabsorbed	1st	2nd	1st	2nd
% S.L.	35.5	14.5	-2.0	42.8	46.0
anti-D titre	1/16	nil	nil	1/16	1/16

* % specific lysis of O R_1R_1 targets with serum pre and post absorption as shown

E/T 5:1 (4 x 10^4 RBC) adherent monocytes removed : 18 hr culture

Table 8.3.2 ABSORPTION OF CYTOLYTIC COMPONENT OF ANTI-D IgG WITH D-POSITIVE (O R_1R_1) RBC

		Absorption with			
		D pos (R_1R_1)		D neg (rr)	
	Unabsorbed	1st	2nd	1st	2nd
** % S.L.	72.5	13.2	2.0	62.0	63.5
anti-D titre	1/32	1/1	nil	1/16	1/16

* IgG III anti-D

** % specific lysis of O R_1R_1 RBC pre and post absorption as shown

E/T 10:1 (4×10^4 RBC); adherent monocytes removed : 18 hr. culture

SECTION II - INVESTIGATION INTO THE MECHANISM OF RBC LYSIS

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1.0 TIME COURSE OF SPECIFIC LYSIS

It has already been shown that significant K-cell lysis has occurred by 2-2½ hours of incubation (section I, fig. 7.2.1, 7.2.2). These results, and others from similar experiments, have been combined to give an overall indication of the kinetics of RBC lysis and are illustrated in fig. 1.0. Significant lysis is seen by one hour and there is a relatively rapid increase in lysis over the next hour. The rate of lysis is then constant, giving a linear increase in % S.L. which can be extrapolated to 100% lysis at approximately 48 hours. This could not be verified experimentally due to the high rate of spontaneous ^{51}Cr release from RBC after 24 hours.

It was noted that there was some individual variation in that maximum lysis was often reached by 18 hours as indicated by a plateau in % specific lysis. In others there was a steady increase from 30 min to 18 hours. The variability can be attributed to differences in K-cell function between the individuals since an E/T of 10:1 was arbitrarily chosen, and in some cases this might have been suboptimal.

The type of time-course of lysis is similar to that reported with K-cell lysis of chicken RBC (Wisløff et al 1974; Calder et al 1974) and a lymphoblastoid cell line (Trinchieri et al 1977) and is probably due to the action of the same effector cell population.

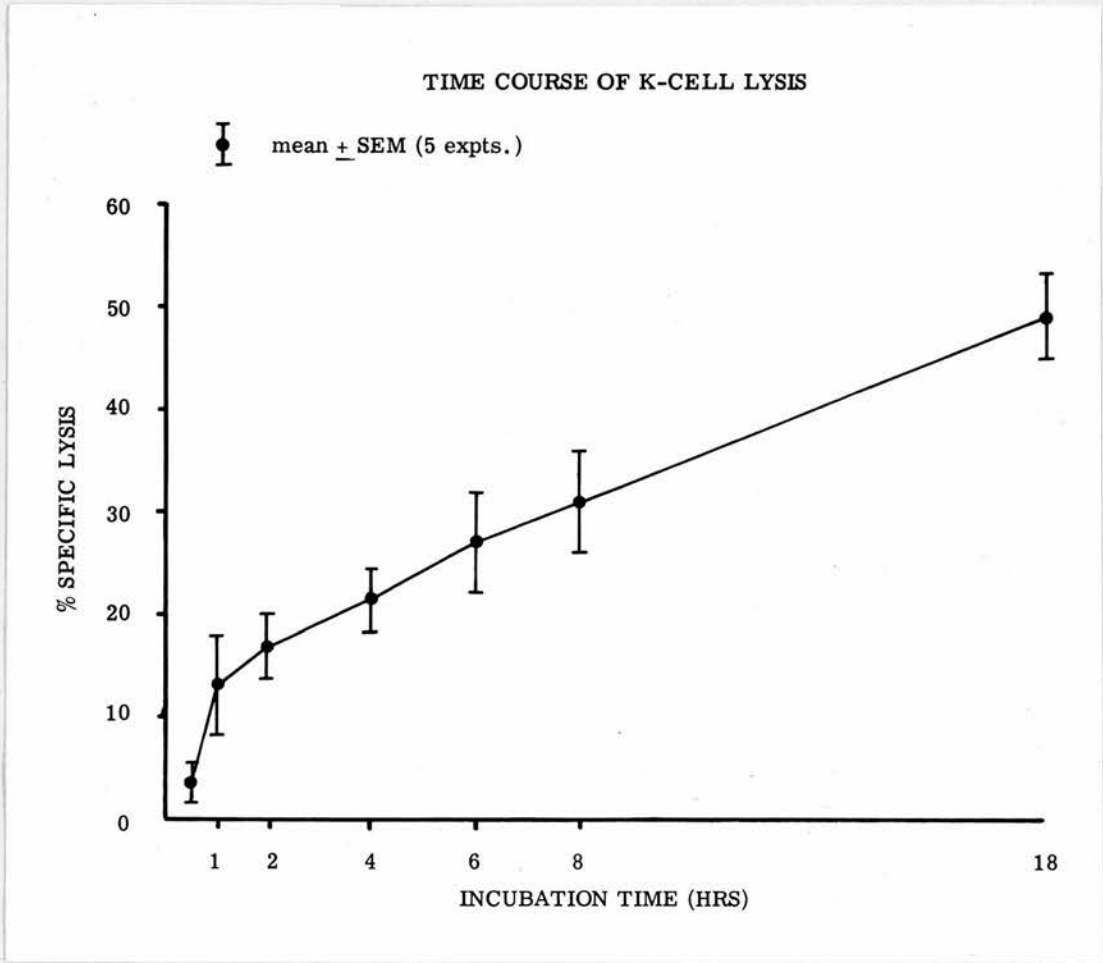
TIME COURSE OF K-CELL LYSIS
mean ± SEM (5 expts.)
% SPECIFIC LYSIS
60
50
40
30
20
10
0
1
2
4
6
8
18
INCUBATION TIME (HRS)

Fig. 1.0

2.0 ENHANCEMENT OF SPECIFIC LYSIS BY CENTRIFUGATION CONTACT

2.1 Alteration of incubation times

It is likely that centrifugation prior to culture allows closer contact between effector and target cells and hence more efficient lysis.

The effects on five individuals studied at various times are shown in fig. 2.1. It can be seen that in all cases except one (donor C) centrifugation significantly enhances the eventual final % specific lysis when compared with settling under gravity after a given incubation time - other conditions of the culture being equal. With donor A there is >100% overall increase over the longer incubation time. A similar phenomenon is seen with B, D and E where the difference between pre-spin/settle is quite marked after 18 hour culture. It is difficult to explain why this should be the case since one might expect that during the longer period of incubation any advantage in contact enhancement would cancel out. The spontaneous ^{51}Cr release from the RBC is not increased by the centrifugation process so the RBC are not likely to be damaged by such a mild centrifugation (which is in any case less than that used during the initial washing stages during the preparation of ^{51}Cr-labelled RBC). It may be that aggregation of RBC is enhanced by this spin, analogous to the easier identification of agglutination in the indirect antiglobulin test where contact centrifugation enables larger agglutinates to form. With larger agglutinates

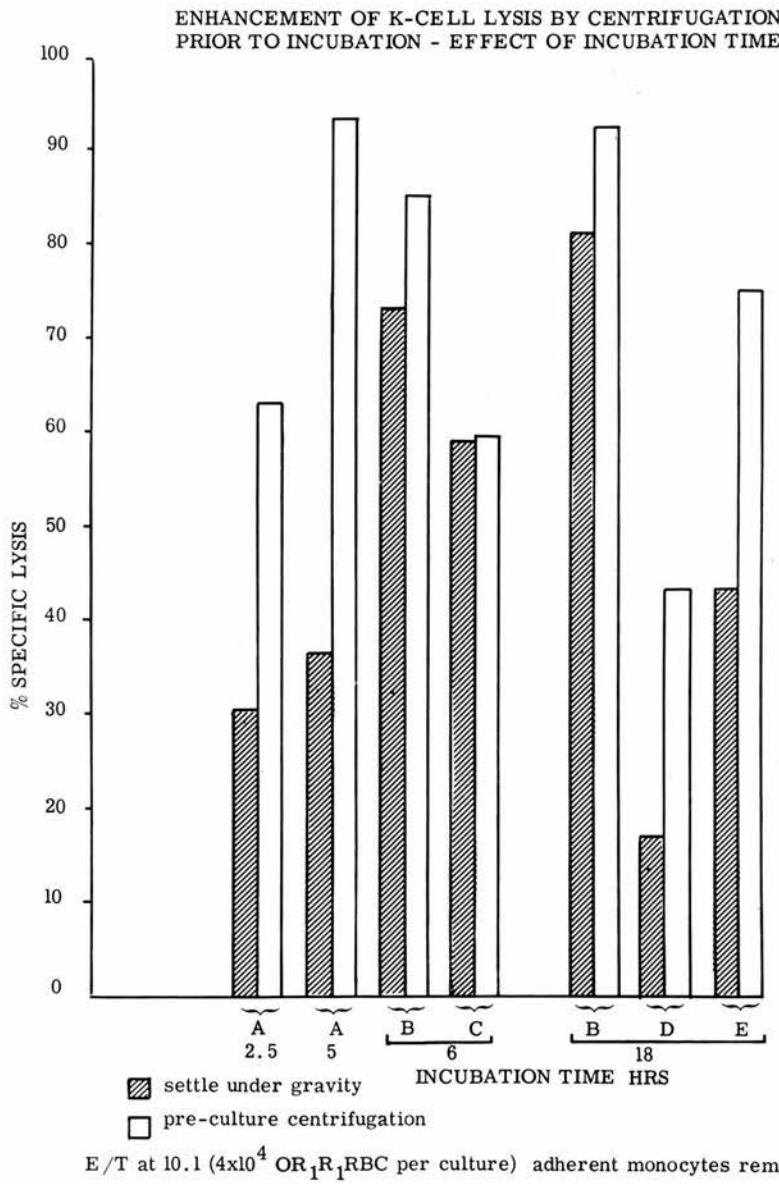
ENHANCEMENT OF K-CELL LYSIS BY CENTRIFUGATION CONTACT PRIOR TO INCUBATION - EFFECT OF INCUBATION TIME
% SPECIFIC LYSIS
100
90
80
70
60
50
40
30
20
10
0
A
2.5
A
5
B
C
6
B
D
E
18
INCUBATION TIME HRS
settle under gravity
pre-culture centrifugation
E/T at 10.1 (4x10^4 OR1R1RBC per culture) adherent monocytes removed

Fig. 2.1

in culture it may be easier for the K-cells to lyse a given number of RBC without having to move too far. This may be relevant to what happens *in vivo* in the spleen where sensitised RBC are in close contact with mononuclear cells in the splenic sinuses enabling efficient lysis of these RBC to take place.

In only one case (donor C) did the centrifugation not enhance ^{51}Cr release at the time studied (see 2.2 below).

2.2 Alteration of E/T ratio

In table 2.2, cells from donor C above were studied at different E/T ratios after 6 hour cultures and it can be seen that there is no difference between pre-spin/settle at the usual 10:1 ratio as already noted in figure 2.1 However, the pre-spin results in higher % specific lysis at the lower ratios of 5:1 and 2:1 where there are less effector cells present in culture. It is likely that in this particular instance the effector K-cells were so active as to show no difference between pre-spin and settle until rate-limiting conditions were produced with lower numbers of effector cells.

2.3 Conclusions

It is likely that direct cell-to-cell contact is required for K-cell lysis to occur and that centrifugation enhances specific lysis by inducing contact. A signifcantly higher % specific lysis is seen at the shorter incubation time which suggests that the delay of 30 min. noted in para 1.0 is due to settling of

Table 2.2 ENHANCEMENT OF K-CELL LYSIS BY CENTRIFUGATION CONTACT PRIOR TO INCUBATION- EFFECT OF ALTERING E/T RATIO

E/T ratio	% specific lysis of O R_1R_1 RBC	
	pre-spin	settle
10:1	59.1	59.5
5:1	55.3	39.2
2:1	35.4	24.6

Donor C, fig. 2.1. Identical conditions except alteration of E/T as above : 6 hr. cultures

effectors and RBC in the culture wells. The initiating events leading up to lysis therefore probably occurs in a matter of minutes of cell contact.

3.0 COMPARISON OF PHAGOCYTOSIS WITH EXTRACELLULAR LYSIS OF RBC

Table 3.0.1 shows that in cultures with adherent monocytes removed, the degree of phagocytosis (as estimated by ^{51}Cr retained by the effector cell population) was negligible since there was no difference between cultures containing sensitised or unsensitised RBC. This indicates that the ^{51}Cr associated with the effector cells was due to non-specific factors ie. RBC membrane fragments contaminating the pellet after RBC lysis. In parallel cultures, the efficiency of the extracellular lytic process was shown by the very high extracellular ^{51}Cr release in the presence of effector cells and anti-D sensitised RBC.

The more detailed experiment in table 3.0.2 shows that there is some phagocytosis if monocyte-rich populations are used, with some 1.9% of RBC-associated ^{51}Cr present in the effector cell pellet after two hours incubation, increasing to 6.2% after 18 hours incubation. There was a greater degree of extracellular lysis at the same incubation times (14.2 and 27.5% respectively). The parallel experiments with monocyte-depleted populations showed much greater extracellular lysis (52.5% after 18 hours) but no greater ^{51}Cr intracellular uptake in the effector cell pellet than that seen with the unsensitised RBC control cultures. It is interesting to note that the monocyte-depleted suspensions are much more efficient at lysing RBC which suggests that the effector cell population is selectively enriched following monocyte depletion.

Table 3.0.1 COMPARISON OF PHAGOCYTOSIS WITH EXTRACELLULAR LYSIS OF RBC

	% ^{51}Cr retained* by effectors incubated with		% ^{51}Cr** release in culture
	unsensitised RBC	anti-D coated RBC	
(1)	7.4	7.7	67.1
(2)	8.8	5.6	73.3
(3)	5.7	5.6	52.5

E/T 10:1 (4×10^4 O R_1R_1 RBC); 18 hr. culture; adherent cells removed

* estimation of RBC phagocytosis by effectors

** estimation of RBC lysis by effectors in presence of anti-D

Louden serum neat for pre-sensitisation

Table 3.0.2 COMPARISON OF PHAGOCYTOSIS WITH EXTRACELLULAR LYSIS OR RBC

incubation time	% ^{51}Cr retained by effectors* incubated with			% ^{51}Cr release in*** culture	
	unsensitised RBC	anti-D coated RBC**			
	pre nylon	pre nylon	post nylon	prc nylon	post nylon
2 hr	6.8	8.7	7.1	14.2	36.8
18 hr	8.7	14.9	7.0	27.5	52.5

* estimation of phagocytosis of ^{51}Cr RBC

** anti-D IgG II used to pre-sensitise RBC

*** estimation of extracellular RBC lysis by effectors in presence of anti-D

E/T 10:1 (4×10^4 O R_1R_1 RBC per culture)

% monocytes (esterase) pre-nylon = 31%

% monocytes (esterase) post-nylon = 0%

4.0 NON-LYSIS OF "BYSTANDER" RBC IN CULTURE

It has been demonstrated above that extracellular lysis is the main mechanism of destruction of the anti-D sensitised RBC. The lytic process is specific in that anti-D target cell antibody is required to sensitise the RBC but is non-specific in that effector cells from any donor will suffice in mediating lysis. Experiments were designed to demonstrate restriction of the lytic process to antibody-coated targets and the results shown in table 4.0. The experimental design involved incubating rhesus positive RBC and rhesus negative RBC in the same cultures under conditions which are known to lyse the D-positive RBC and to observe whether or not any lysis of the bystander D negative RBC occurred. The controls, 1-4, show that the anti-D is not directly toxic to either Rh positive (1) or Rh. negative RBC (2) and there is no direct effector cell lysis of either Rh. positive (3) or negative RBC (4) in the absence of anti-D. Experiment 5 demonstrates that there is good RBC lysis in cultures containing effector cells, D positive RBC and anti-D. Experiment 7 shows that the addition of unlabelled D negative RBC has no effect on the degree of lysis achieved. Experiment 6 shows that effector cells with anti-D are not effective in lysing D negative RBC and experiment 8 shows that as D positive RBC are being lysed (as in 7 but with unlabelled RBC) there is no "bystander" lysis of D negative RBC which are also present in culture.

Table 4.0 NON-LYSIS OF BYSTANDER RBC IN CULTURE

	Cultures containing:-						% ^{51}Cr release
	Effectors	0 $R_1R_1^o$	0 $R_1R_1^*$	0 rr^o	0 rr*	anti-D	
1.	-	-	+	-	-	+	1.5
2.	-	-	-	-	+	-	0.5
3.	+	-	+	-	-	-	2.7
4.	+	-	-	-	+	-	0.5
							% specific lysis
5.	+	-	+	-	-	+	80.1
6.	+	-	-	-	+	+	0.3
7.	+	-	+	+	-	+	87.2
8.	+	+	-	-	+	+	1.4

o = unlabelled RBC * = ^{51}Cr labelled RBC 1-4 controls ; 5-8 test

- and + indicate absence or presence in culture

4 x 10^4 ^{51}Cr RBC per culture giving E/T 10:1 ; for 7 & 8 an additional 4 x 10^4 unlabelled RBC was added, but maintaining an E/T of 10:1 with respect to the D pos. RBC

Since the D positive and D negative RBC are intimately mixed with effector cells in culture it is apparent that there is no non-specific release of a lytic factor into the culture medium (as is the case with lymphocytotoxins produced by mitogen-stimulated T lymphocytes, Hiserodt & Granger, 1976). It is likely that a lytic molecule is produced in response to the stimulus of target-bound antibodies and which is effective only over a very short distance before being either bound to the target cell membrane or inactivated in the culture medium. This probably explains why the effector cells do not destroy their own membranes since it is known that lymphoid cell surface membranes are not intrinsically resistant to K-cell lysis provided that they are sensitised by an appropriate antibody (Rachelefsky et al 1975; Kovithavongs et al 1974).

4.1 Non-transfer of lysis by culture supernatants

The absence of a non-specific lytic factor produced during K-cell lysis of RBC was confirmed by supernatant transfer experiments (table 4.1). Supernatants from cultures which had active K-cells, as demonstrated by high % specific lysis, were no more effective in inducing further lysis of RBC in culture than were the control culture where lysis had not originally occurred.

Table 4.1 NON-TRANSFER OF LYSIS BY K-CELL CULTURE SUPERNATANTS

		% specific lysis with	
		K cell cultures	transferred* supernatant
1.	E + RBC + anti-D	42.3	8.7
	E + RBC + AB serum	-1.2	-0.2
2.	E + RBC + anti-D	63.2	2.2
	E + RBC + AB serum	-2.5	1.5

* supernatants from replicate cultures transferred to fresh RBC culture in absence of effector cell

E/T ratio 10:1 (4×10^4 0 R_1R_1 RBC); adherent monocytes removed; 18 hr. cultures

5.0 ESTIMATION OF K-CELL EFFICIENCY

Since it is likely that the effector K-cell is only a minority population of peripheral blood mononuclear cells, at the most 10-15%, and according to some estimates, in the region of 1-2% (Biberfield et al 1975), it is likely that these K-cells must be capable of causing the lysis of more than one target RBC.

In the following experiments, a constant number of mononuclear cells were added per culture and the absolute number of RBC was varied, a reversal of the usual procedure. Assuming that the $\%^{51}Cr$ release in culture accurately reflects the percentage of RBC lysed, then the numbers of red cells lysed per culture can be calculated. The experimental results are shown in table 5.0. It can be seen that as more RBC become available, the actual number of RBC lysed also increases although the percentage of RBC lysed of course falls. The numbers of RBC lysed per putative K-cell in culture has been calculated on the basis that the mononuclear suspension contains either 10% K-cells or 1% K-cells. In either case, it can be seen that the effector cells are able to lyse increasing numbers of RBC and at the 1% level they appear to be able to lyse in the region of 40 RBC during the course of incubation. Assuming that as many as 10% of the added cells were K-cells, approximately 4 RBC could be lysed per effector cell. Since close contact is required between effector cell and RBC, the effector cells must be motile in order to achieve the lysis of 40 RBC. Further evidence to

support this statement is presented in the section dealing with metabolic inhibitors.

Table 5.0 ESTIMATE OF NUMBERS OF RBC LYSED PER EFFECTOR CELL

RBC targets per culture x 10^4	Estimate of		No. of RBC lysed***	
	% RBC lysis*	numbers RBC lysed**	10% K-cells	1% K-cells
1 (10/1)+	76.5	7.650	0.8	7.7
2 (5/1)	37.9	7,580	0.8	7.6
4 (2.5/1)	36.7	14,680	1.5	14.7
10 (1/1)	17.7	17,700	1.8	17.7
50 (1/5)	8.3	41,500	4.2	41.5

Constant number (1 x 10^5) of mononuclear cells per culture.

* from % ^{51}Cr release in presence of effectors and anti-D corrected for spontaneous ^{51}Cr release.

** ie. 76.5% of 1 x 10^4 etc.

*** numbers of RBC lysed per culture assuming 10% or 1% of 1 x 10^5 are K-cells + E/T ratio.

6.0 NON-CYTOPHILIC ANTIBODY EFFECT OF ANTI-D

The effect of pre-incubation of mononuclear effector cell suspensions pre and post monocyte depletion is shown in table 6.0.1 and table 6.0.2 where both the anti-D containing serum and the IgG fraction prepared from it, were used. The experiments were designed to show a cytophilic effect of anti-D in that this antibody could have bound via the Fc fragment to the Fc receptor on the effector cell and then "transfered" with the effector cells into culture and hence result in target cell lysis. It can be seen that even in those cultures containing substantial numbers of monocytes (17.1%, and 17.7%) there is no evidence of a cytophilic transfer of anti-D and only background ^{51}Cr release is obtained when these effector cells are incubated with RBC. It may be that the minimal washing steps to remove unbound anti-D results in the loss of any effector-cell-bound antibody due to the low binding affinity, or alternatively, target cell antibody will not bind to the effector cells until the Fc fragment has been "activated" following conformational changes after interaction with the red cell target antigens. The above results tend to confirm that target cell antibody must first bind to the target cell via the Fab fragment before lysis can be induced.

6.0.1 Effect of pre-incubation of effector cells with anti-D serum (Louden)

	% specific lysis
E + RBC + anti-D serum	42.1
E + RBC + AB serum	-11.5
E* + RBC + AB serum	1.03
E** + RBC + AB serum	-0.1

* effectors pre-incubated with anti-D pre-nylon passage (17.1% monocytes)

** effectors pre-incubated with anti-D post-nylon passage (1.3% monocytes)

E/T 10:1 (4×10^4 O R_1R_1 RBC)

18 hr. culture

6.0.2 Effect of pre-incubation of effector cells with anti-D IgG III

		% specific lysis
E(1)	+ RBC + anti-D	90.7
E(1)	+ RBC + AB serum	-0.5
E(1)*	+ RBC + AB serum	-0.9
E(1)**	+ RBC + AB serum	0.9
E(2)	+ RBC + anti-D	86.5
E(2)	+ RBC + AB serum	0.4
E(2)*	+ RBC + AB serum	-0.1
E(2)**	+ RBC + AB serum	0.73

E(1)/(2)* Effectors pre-incubated with anti-D (pre-nylon column) (1) 12.5% monocytes (2) 17.7% monocytes

E(1)/(2)** Effectors pre-incubated with anti-D (post nylon column) (1) 1.5% monocytes (2) 0.7% monocytes

E/T 10:1 (4×10^4 RBC per culture); 18 hr culture

7.0 EFFECTS OF METABOLIC INHIBITORS

7.1 Introduction

By the use of inhibitors of various stages of metabolic function in intact cells, some indication of the cellular events involved in K-cell lysis can be obtained. The following experiments detail the results obtained with inhibitors of DNA replication (mitomycin C), RNA synthesis (actinomycin D), protein synthesis (puromycin), metabolism of glucose (2-deoxyglucose), microtubule function (colchicine), phagocytosis and microfilament function (cytochalasin B) and lysosome membrane stability (hydrocortisone).

Some experiments were done both pre and post nylon column removal of monocytes in order to see whether any difference in the pattern of inhibition could be obtained. A more extended range of experiments were done on monocyte-depleted suspensions with a greater variety of metabolic inhibitors. In each case the concentrations used had been checked for non-toxic effects on the RBC and the effector cells. In order to use a system with maximum sensitivity, red cells pre-sensitised for anti-D were used and it should be noted that the results are expressed as percent cytotoxic activity (see methods section) where the % S.L. with inhibitor is related to the % S.L. in the absence of inhibitor (100% value); in this way the results obtained with different individuals can be compared.

7.2 Results

7.2.1 2-deoxyglucose

The inhibitory effect of a dose range from 1-50 mM is seen in fig. 7.2.1, in this case, monocyte-depleted cultures were used. There is a dose-dependent inhibition to approximately 50% at the highest concentration of 50 mM. However, quite large individual variations were recorded with virtually 100% inhibition at 20 mM in one individual and only 10% inhibition at 50 mM in another.

7.2.2 Mitomycin C

The results of one experiment with monocyte-depleted suspensions are shown in fig. 7.2.2 and it can be seen that at the highest concentration of 25 µg/ml only 15% inhibition was seen. With another individual (results not shown) there was no difference in the % cytotoxic activity between 5 and 30 µg/ml.

7.2.3 Actinomycin D

The results of the effects of a range of concentrations from 0.5 - 10 µg/ml are shown in fig. 7.2.3. Monocyte-depleted cultures were used and there is a dose-dependent inhibition to a maximum of 30% at 5 µg/ml and very little further inhibition is seen even up to 10 µg/ml.

7.2.4 Puromycin

The results of inhibition with 0.5-10 µg/ml are shown in fig. 7.2.4. Monocyte depleted suspensions were used and it can be seen that there is an initial dose-dependent inhibition to approximately 50% at 5 µg/ml

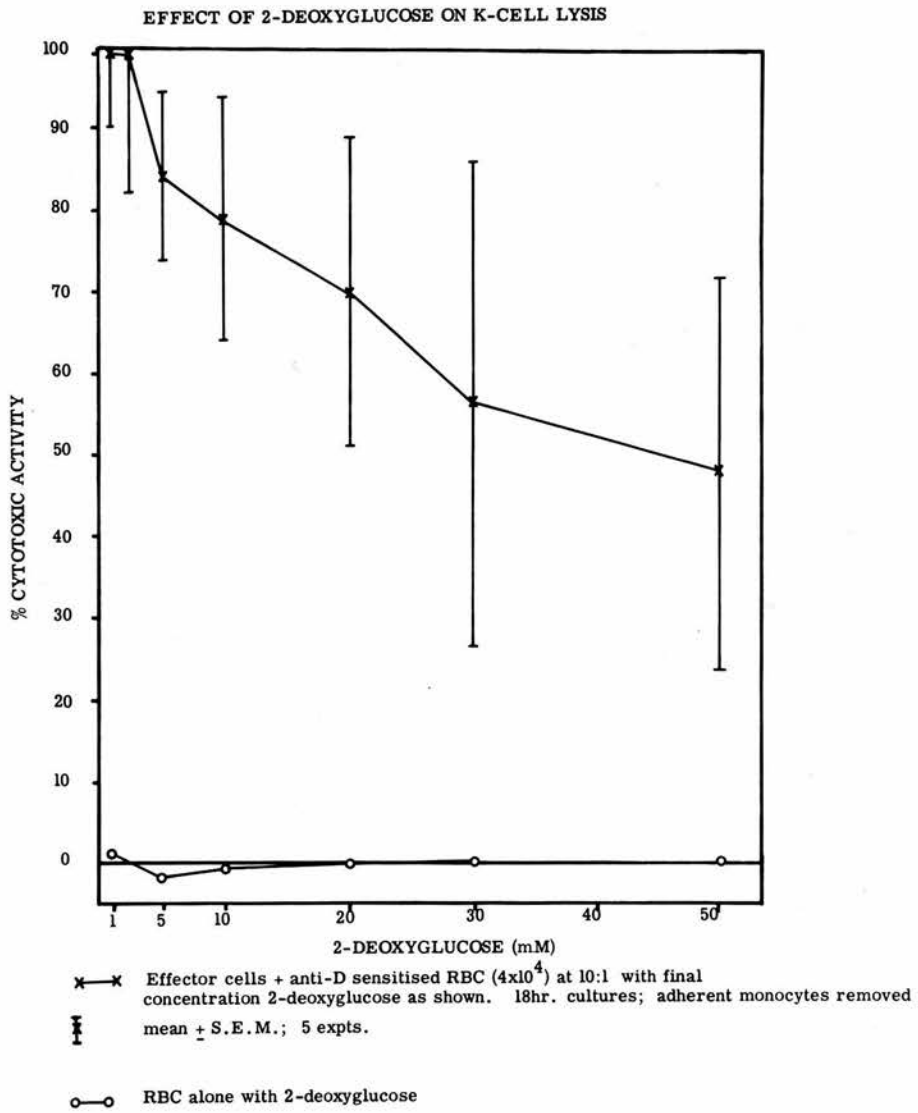
EFFECT OF 2-DEOXYGLUCOSE ON K-CELL LYSIS
% CYTOTOXIC ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
1
5
10
20
30
40
50
2-DEOXYGLUCOSE (mM)
Effector cells + anti-D sensitised RBC (4x10^4) at 10:1 with final concentration 2-deoxyglucose as shown. 18hr. cultures; adherent monocytes removed
mean ± S.E.M.; 5 expts.
RBC alone with 2-deoxyglucose

Fig. 7.2.1

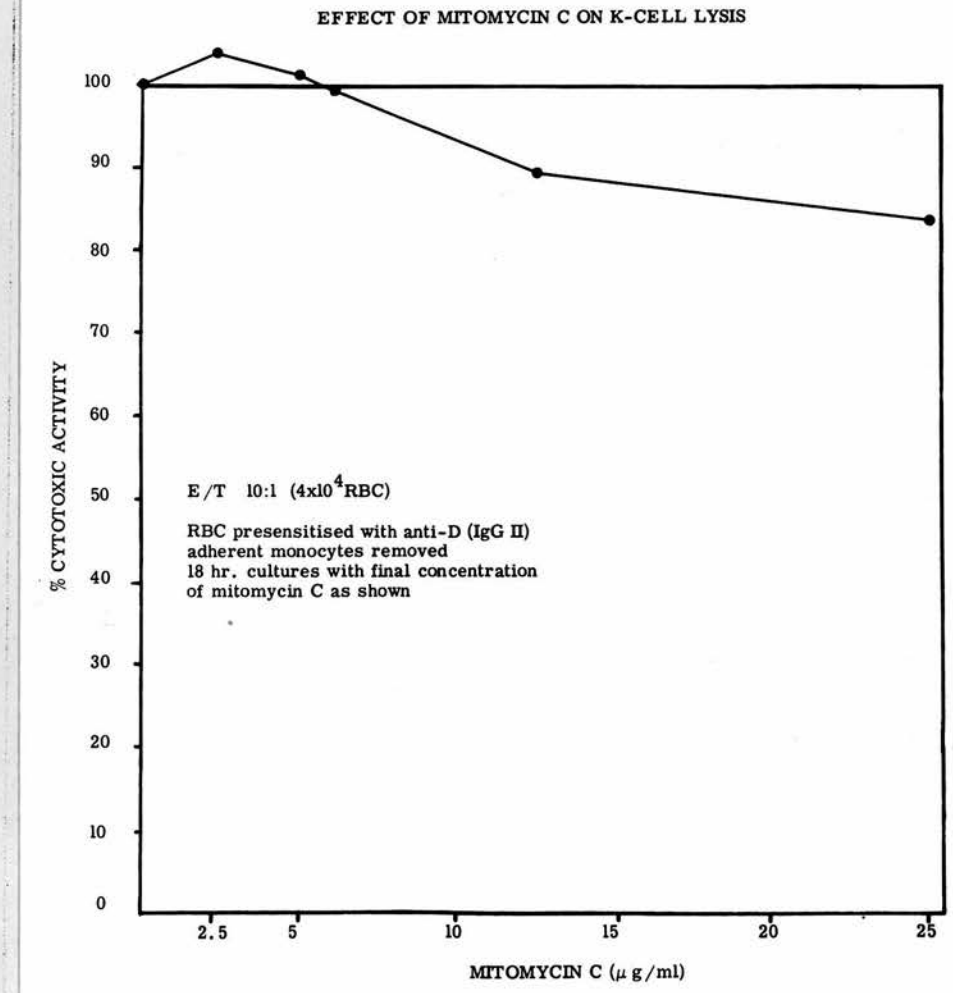
EFFECT OF MITOMYCIN C ON K-CELL LYSIS
% CYTOTOXIC ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
E/T 10:1 (4x10^{4}RBC)
RBC presensitised with anti-D (IgG II)
adherent monocytes removed
18 hr. cultures with final concentration
of mitomycin C as shown
2.5
5
10
15
20
25
MITOMYCIN C (μg/ml)

Fig. 7.2.2

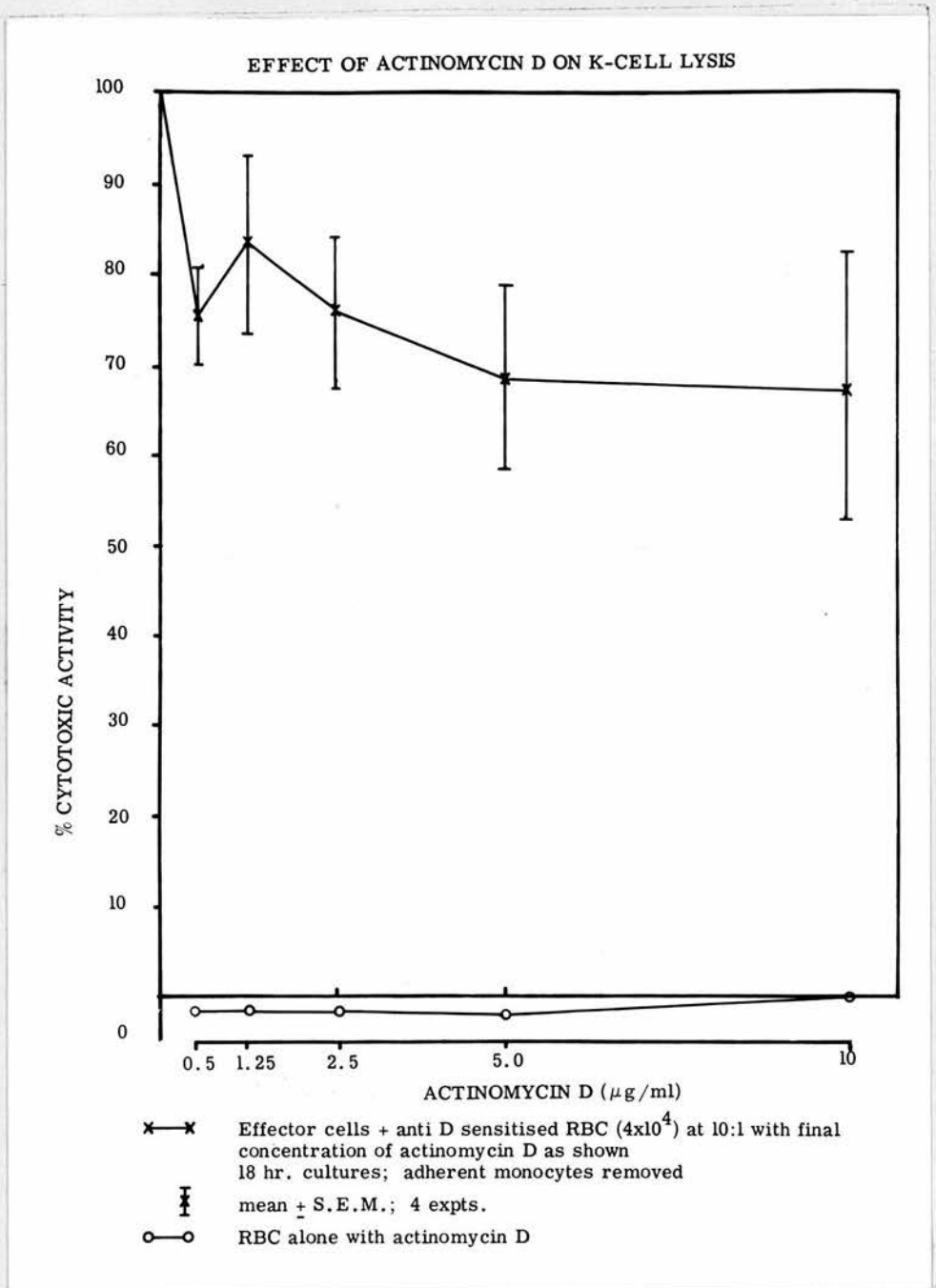
EFFECT OF ACTINOMYCIN D ON K-CELL LYSIS
% CYTOTOXIC ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
0.5
1.25
2.5
5.0
10
ACTINOMYCIN D (μg/ml)
Effector cells + anti D sensitised RBC (4x10^4) at 10:1 with final concentration of actinomycin D as shown
18 hr. cultures; adherent monocytes removed
mean ± S.E.M.; 4 expts.
RBC alone with actinomycin D

Fig. 7.2.3

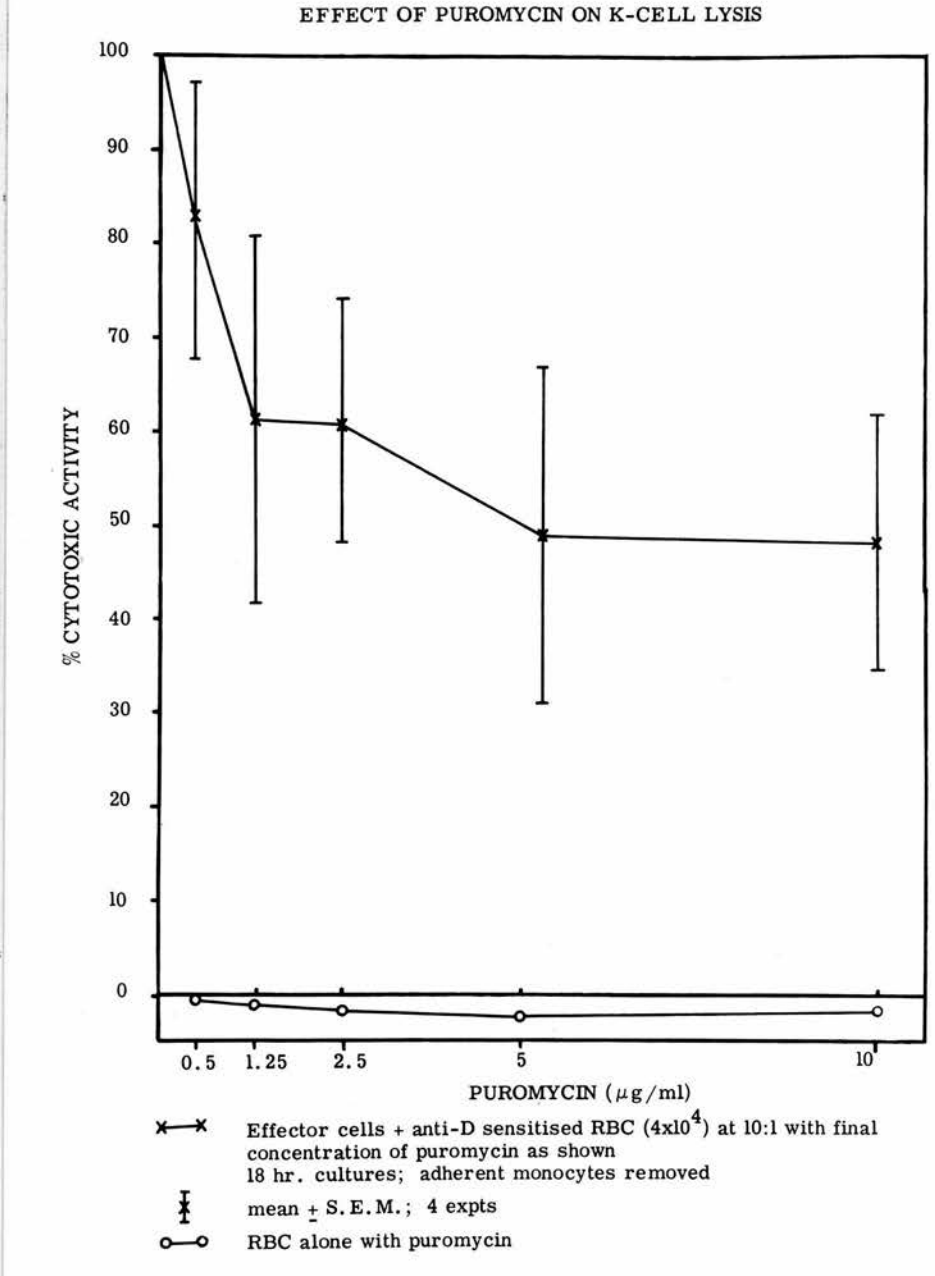
EFFECT OF PUROMYCIN ON K-CELL LYSIS
% CYTOTOXIC ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
0.5
1.25
2.5
5
10
PUROMYCIN (μg/ml)
Effector cells + anti-D sensitised RBC (4x10^4) at 10:1 with final concentration of puromycin as shown
18 hr. cultures; adherent monocytes removed
mean ± S.E.M.; 4 expts
RBC alone with puromycin

Fig. 7.2.4

and very little further inhibition is seen even up to 10 µg/ml.

7.2.5 Colchicine

The results of three experiments with and without removal of the monocytes are shown in figure 7.2.5/1 and a more detailed investigation of the effects of a range of colchicine concentrations on monocyte-depleted cell suspensions are shown in fugure 7.2.5/2. Most interestingly, the pre and post-nylon cultures show significant differences in the degree of inhibition above 0.1 mM, with the monocyte-depleted cultures being the more "resistant" to inhibition at a comparable colchicine concentration - 50% inhibition is seen at approximately 0.8-0.9 mM colchicine in the monocyte-depleted suspensions and at 0.5 mM colchicine in monocyte-rich suspensions. The extended series with monocyte-depleted cultures show comparable dose-dependent inhibition curves with approximately 80% inhibition at 2 mM and 93% inhibition with the highest non-toxic dose of colchicine, 5 mM. Little inhibition is seen up to 0.1 mM but thereafter there is a rapid dose-dependent inhibition of lysis.

7.2.6 Hydrocortisone

The results of a range of concentrations tested with and without removal of monocytes is seen in figure 7.2.6/1 and a more extended series of experiments on monocyte-depleted cultures in figure 7.2.6/2.

Again, cultures show a dose-dependent inhibition

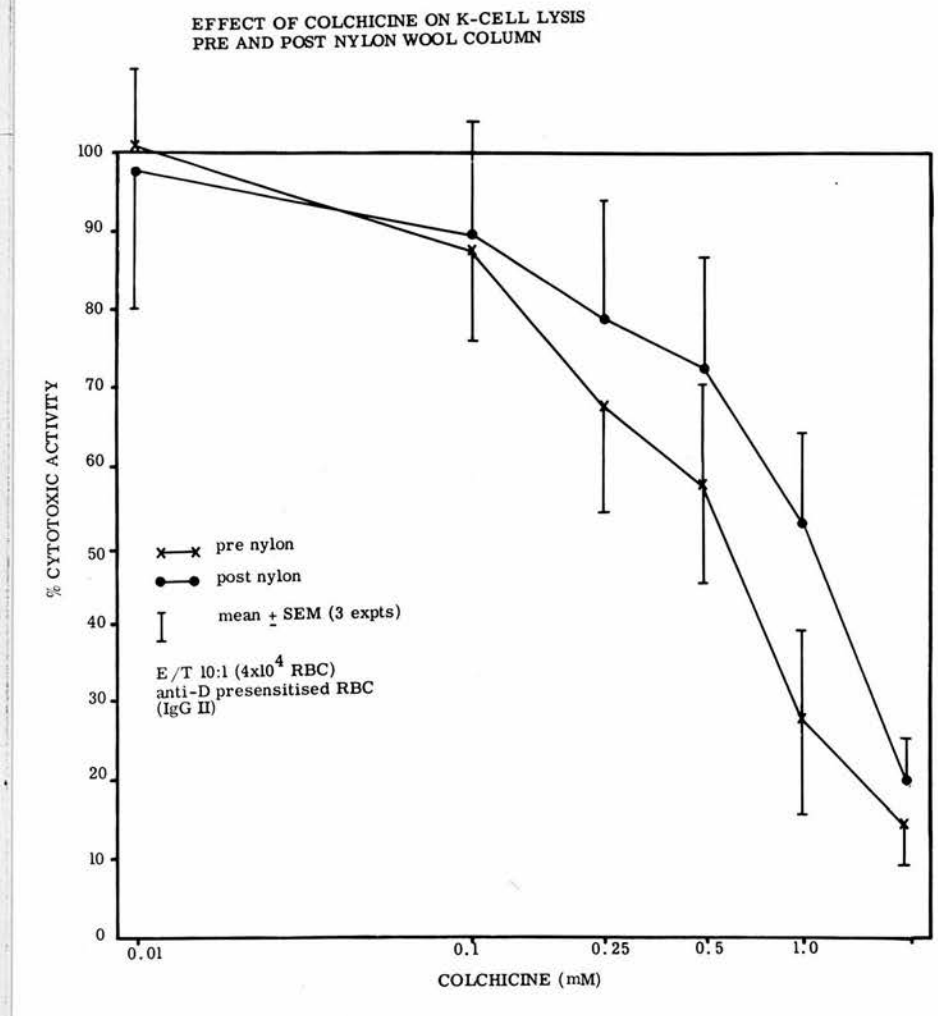

Fig. 7.2.5/1

paired t test pre vs. post

at 0.1 mM	not significant
at 0.25 mM	not significant
at 0.5 mM	$p < 0.10 > 0.05$
at 1.0 mM	$p < 0.05 > 0.025$
at 2.0 mM	$p < 0.0025 > 0.0005$

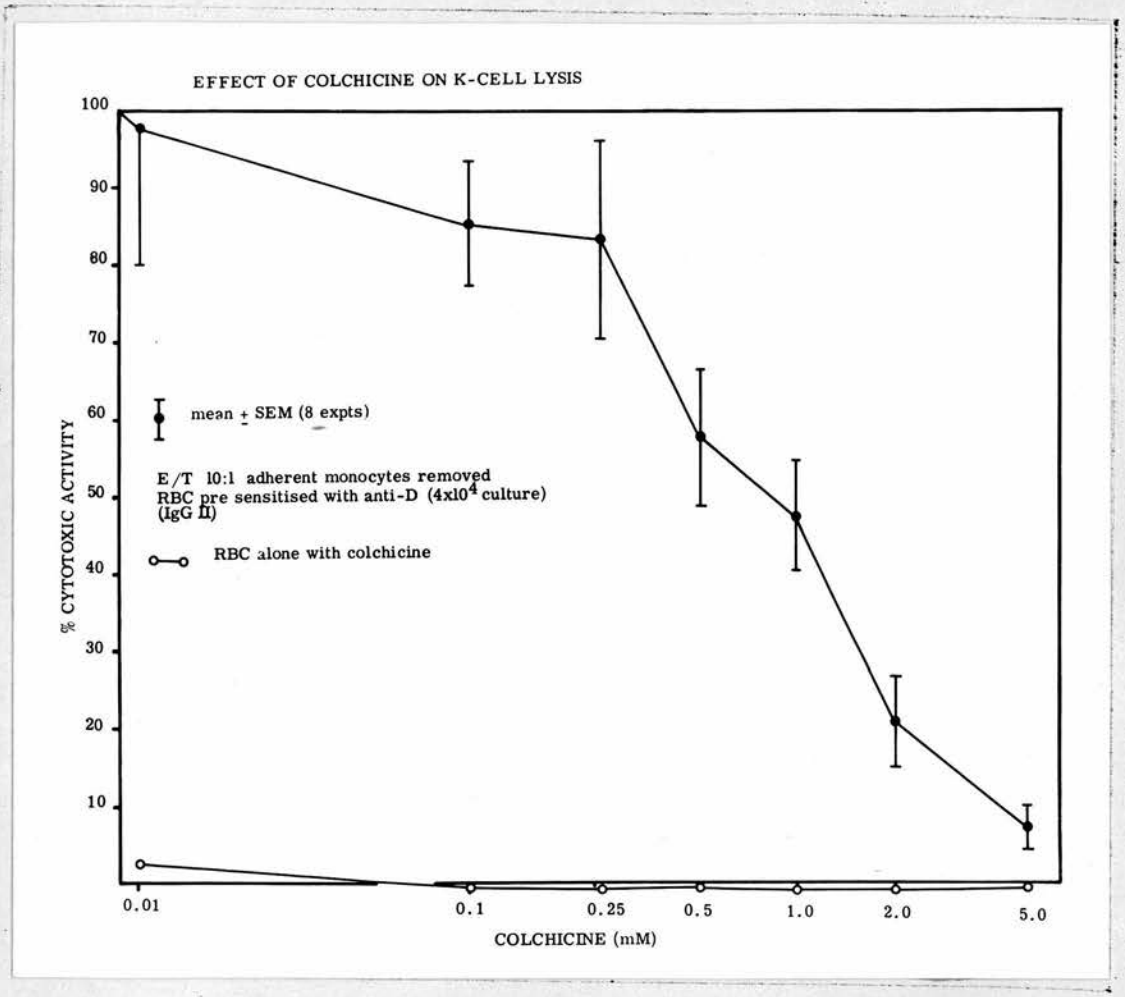
EFFECT OF COLCHICINE ON K-CELL LYSIS
% CYTOTOXIC ACTIVITY
100
90
80
70
60
50
40
30
20
10
mean ± SEM (8 expts)
E/T 10:1 adherent monocytes removed
RBC pre sensitised with anti-D (4x10⁴ culture)
(IgG II)
RBC alone with colchicine
0.01
0.1
0.25
0.5
1.0
2.0
5.0
COLCHICINE (mM)

Fig. 7:2.5/2

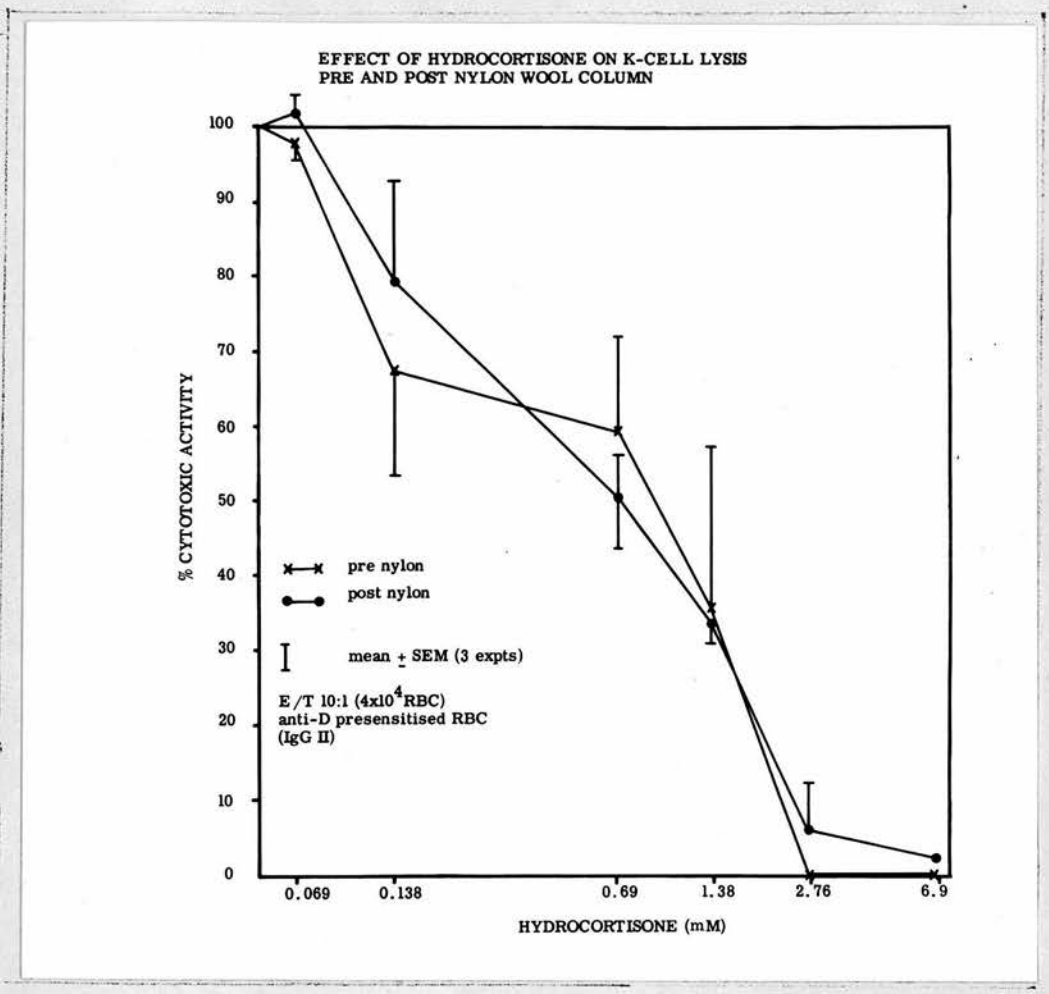

Fig. 7.2.6/1

paired t test : pre vs. post

at 0.069 mM not significant

at 0.138 mM $p < 0.0025 > 0.0005$

at other concentrations not significant

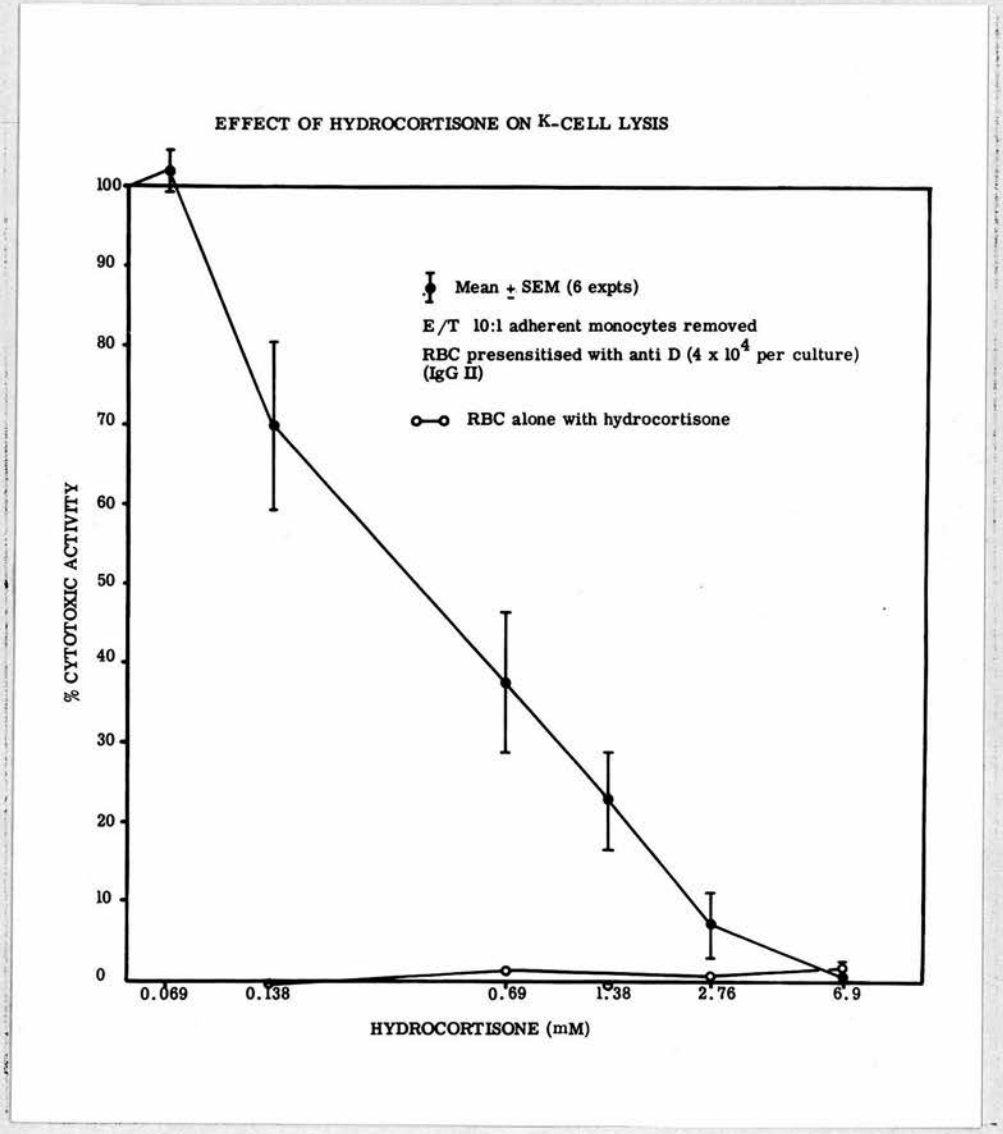
EFFECT OF HYDROCORTISONE ON K-CELL LYSIS
Mean ± SEM (6 expts)
E/T 10:1 adherent monocytes removed
RBC presensitised with anti D (4 x 10^4 per culture) (IgG II)
RBC alone with hydrocortisone
% CYTOTOXIC ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
0.069
0.138
0.69
1.38
2.76
6.9
HYDROCORTISONE (mM)

Fig. 7.2.6/2

of RBC lysis with the pre and post nylon series being comparable. In this case, there is very little difference between the pre and post-nylon cultures except at 0.138 mM where again monocyte-depleted cultures are more resistant to inhibition. Complete inhibition of red cell lysis is seen at 6.9 mM with 94% inhibition at 2.76 mM in the monocyte-depleted cultures.

In the experiments done concurrently on pre and post-nylon suspensions, 50% inhibition occurs at 0.69 mM post-nylon and approximately 0.9 mM pre-nylon.

7.2.7 Cytochalasin B

The effects of a range of concentrations from 0.025 to 2.5 µg/ml, tested pre and post monocyte depletion, are shown in fig. 7.2.7/1 and a more extended range with monocyte depleted cultures are shown in fig. 7.2.7/2.

In this instance, there is a markedly different effect with cytochalasin B pre and post depletion. There appears to be stimulation of monocyte-rich cultures from 0.025 - 0.5 µg/ml, followed by dose-dependent inhibition above this concentration, and inhibition is almost complete (94%) at 2.5 µg/ml. 50% inhibition is seen at approximately 1 µg/ml. The monocyte depleted cultures show a dose-dependent inhibition throughout the range of dilutions, with virtually complete inhibition (92-94%) at 2.5 µg/ml. 50% inhibition is seen at approximately 0.6-0.7 µg/ml.

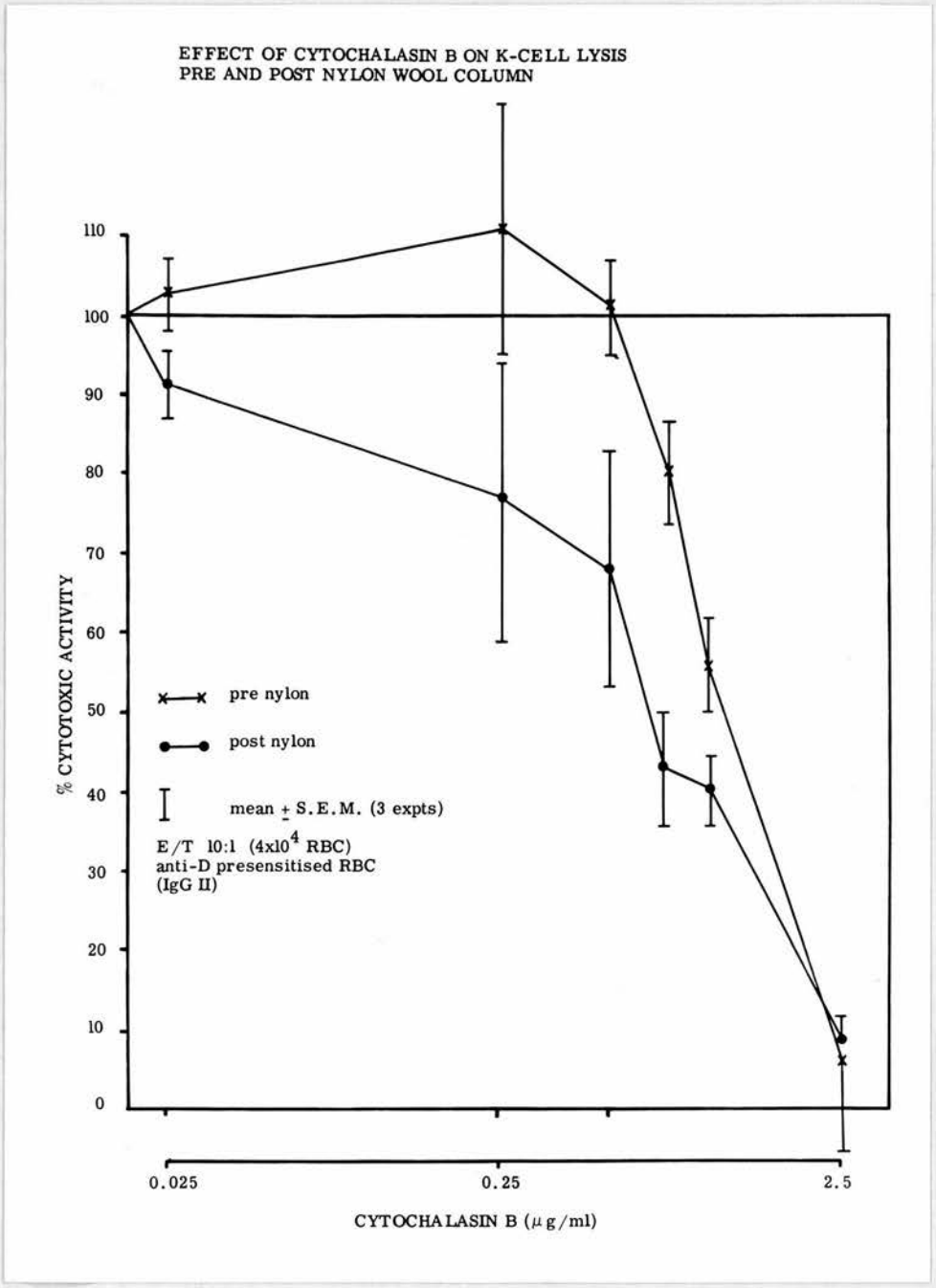

Fig. 7.2.7/1

paired t test pre vs. post

at 0.025 μg/ml	p <0.01 >0.0025
at 0.25 μg/ml	p.<0.05 >0.025
at 0.5 μg/ml	p <0.10 >0.05
at 0.75 μg/ml	p <0.025 >0.0125
at 1.0 and 2.5 μg/ml	not significant

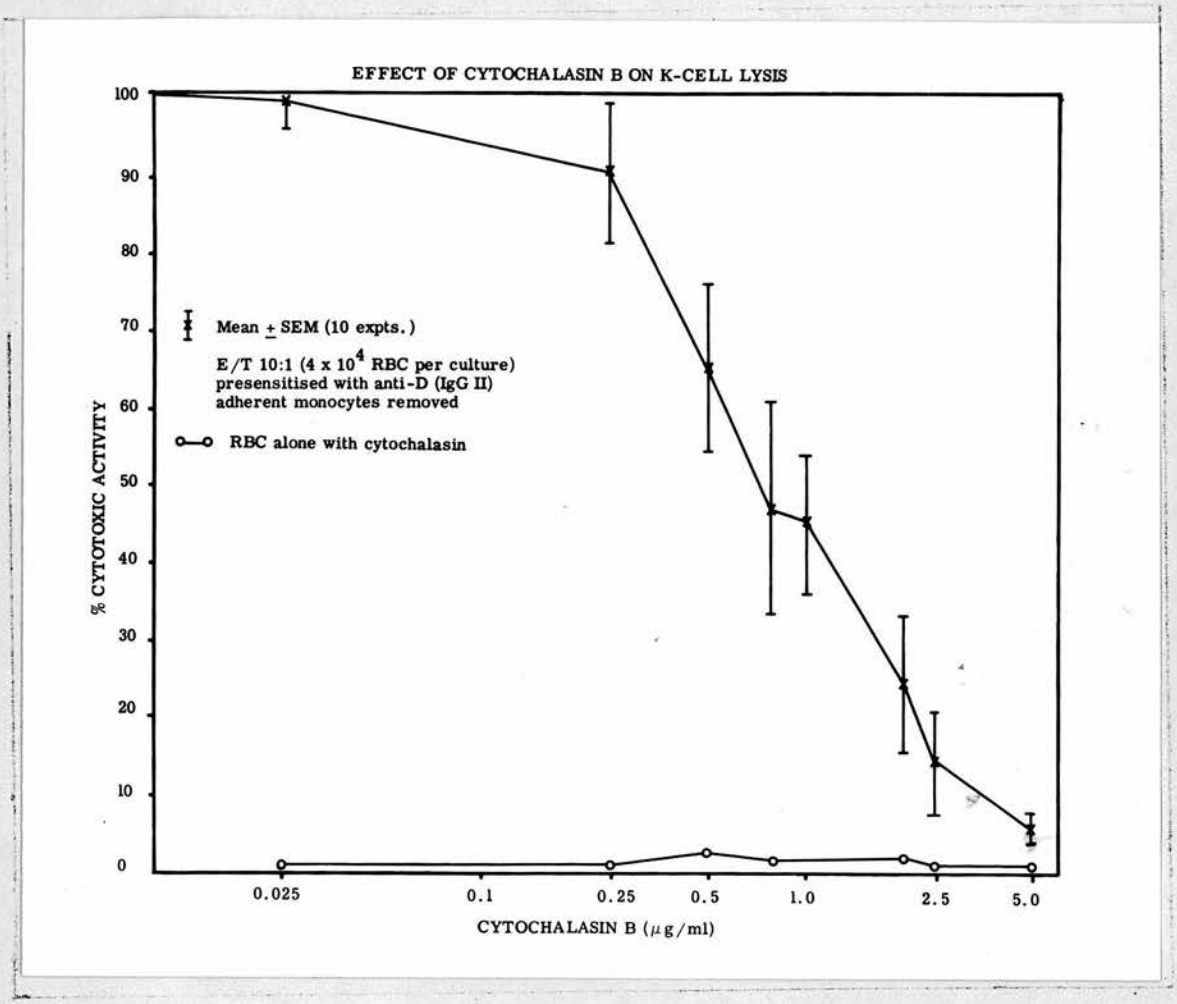
EFFECT OF CYTOCHALASIN B ON K-CELL LYSIS
% CYTOTOXIC ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
Mean ± SEM (10 expts.)
E/T 10:1 (4 x 10^4 RBC per culture)
presensitised with anti-D (IgG II)
adherent monocytes removed
RBC alone with cytochalasin
0.025
0.1
0.25
0.5
1.0
2.5
5.0
CYTOCHALASIN B (μg/ml)

Fig. 7.2.7/2

The differences between pre and post nylon cultures at the lower dilutions are statistically significant (see fig. 7.2.7/1).

7.3 Discussion and conclusions

The effects of a variety of metabolic inhibitors on other K-cell systems have been reported in the literature (MacDonald & Bonnard 1975; Strom et al 1975). The present findings are largely in agreement with the published results.

2-deoxyglucose inhibits phosphoglucoisomerase, an enzyme necessary for the metabolism of glucose via the glycolytic pathway (Wiek et al 1957). Approximately 50% inhibition of specific lysis was seen up to 50 mM 2-deoxyglucose and it is possible that complete inhibition would have been seen at higher concentrations had it been possible to test this experimentally. Unfortunately the RBC depends almost entirely on the metabolism of glucose for the production of an energy source in the form of ATP and 2-deoxyglucose concentrations above 50 mM proved to be toxic to RBC. The present data suggests however that a metabolically effective cell is required for K-cell lysis to occur.

No inhibition was seen with mitomycin C, an agent which cross-links DNA strands and prevents replication and cell division (Sung 1972). These events are therefore not necessary (as they are for blast trans-

formation and possibly for T cell killing) for K-cell lysis. This is in accord with current views on K-cell action.

Actinomycin D inhibits DNA-dependent RNA polymerase (Sung 1972) and therefore prevents RNA synthesis - in this case possibly messenger RNA synthesis. Up to 30% inhibition of K-cell lysis is seen over the dose-range tested. It is possible that some m-RNA synthesis de novo is required for effective K-cell lysis to occur, but that inhibition is incomplete due to the presence of pre-formed m-RNA able to "direct" synthesis of protein.

Puromycin inhibits protein synthesis by interfering with translation at the ribosome level (Watanabe, 1972). Approximately 50% inhibition is seen at the highest dose tested and again it is possible that pre-formed protein molecules are able to effect K-cell lysis up to a point but that de novo synthesis is further prevented by puromycin.

Colchicine disrupts microtubule function which is necessary for the export of protein (and other) molecules synthesised within the cell to the exterior (Weisenberg et al 1968). Complete inhibition of K-cell lysis can be produced with concentrations of 5 mM colchicine and there is a dose-dependent inhibition below this level. It is possible that a cytotoxic molecule is being "exported" to the cell surface and that the

export is inhibited by colchicine. It is of interest that the inhibition curves for suspensions with and without monocytes indicate the monocyte-depleted suspensions are more "resistant" to colchicine inhibition. This tends to suggest that either monocytes are not active in this particular system (since one would expect more and not less inhibition with fewer monocytes present in culture) or that the cytotoxic mechanism differs between the monocyte and the K-cells.

One of the many effects of hydrocortisone is thought to be stabilisation of lysosome membranes (Bitensky et al 1974) and there is a marked dose dependent inhibition of specific lysis with complete inhibition at 6.9 mM. If the "lytic" factor is present intracellularly as a lysosome-like packet then this would explain the dose dependent inhibition by hydrocortisone.

Cytochalasin B disrupts microfilament function and hence cell membrane motility and the ability to adhere and phagocytose (Wessels et al 1971). It has also been suggested that it results in the release of lysosomal enzymes from cells (Zurier et al 1973). It is interesting to note that there is enhancement of lysis in the presence of low concentrations of cytochalasin B in monocyte-rich cultures, which could be explained on this basis. With high concentrations, there is a dose-dependent inhibition of specific lysis which is virtually complete at 5 µg/ml. In monocyte-depleted cultures there is a dose-dependent inhibition of K-cell lysis which is again virtually complete at 5 µg/ml. It has been shown that cell contact and "gripping" of the

target is necessary for K-cell lysis (Biberfield et al 1971; Roitt et al 1976), and that motility is required for maximum lysis of dispersed targets in culture. The difference in the dose-dependent inhibition curves can be explained in terms of the initial release of lysosomal contents from monocytes in the presence of low concentrations of cytochalasin B, that this does not occur in the absence of monocytes, and that the dose-dependent inhibition seen at the higher concentrations is a reflection of inhibition of the K-cell population.

In summary one may conclude that the K-cell lytic process require a metabolically active (partial inhibition by 2-deoxyglucose) which should have intact cell motility and membrane motility (inhibition by cytochalasin B), that DNA synthesis is not required (no inhibition by mitomycin C), but some m-RNA synthesis may be required (partial inhibition by actinomycin D) which in turn results in synthesis of a lytic protein molecule (partial inhibition by puromycin). These molecules may be transported to the cell surface by a microtubule system (inhibition by colchicine) in the form of lysosomal organelles (inhibition by hydrocortisone). These lytic molecules are then released at the cell surface which is held in intimate contact by a "gripping" process involving membrane contact (inhibition by cytochalasin B) and these lytic molecules are then responsible for the irreversible damage of the target cell membrane.

8.0 REQUIREMENT FOR DIVALENT CATIONS (Mg^{2+}, Ca^{2+})

It has been shown that divalent cations (predominantly Ca^{2+} and Mg^{2+}) are required for T cell cytotoxicity and for K-cell cytotoxicity although it appears that there are certain differences in detail between the two mechanisms which can distinguish them (Goldstein & Gomperts 1975; Goldstein & Smith 1976). Goldstein & Smith 1976 have shown that mouse spleen cells and human blood effector cells will lyse sensitised sheep RBC in the presence of Mg^{2+} alone (ie. no Ca^{2+}) and that a subpopulation of human effector cells could lyse sensitised SRBC in the absence of either cation.

The effects of the chelating agents EDTA (binds Ca^{2+} and Mg^{2+}) and EGTA (binds Ca^{2+} only) were studied in the K-cell system developed and the results shown in table 8.0.1 and 8.0.2.

In table 8.0.1, a concentration of 0.2 mM EDTA inhibits specific lysis by 59.9%, and EGTA by 45.3% whereas at 2 mM there is virtually complete inhibition with both EDTA and EGTA at 97.1% and 92.7% inhibition respectively. The inhibition with EDTA confirms the cation requirement for lysis. EGTA binds only Ca^{2+} and not Mg^{2+} but there is still a considerable degree of inhibition at 0.2 mM which suggests that Mg^{2+} alone in insufficient in this particular K-cell assay. It may be that there was insufficient Mg^{2+} in culture for maximum lysis since the only source was the AB serum supplement and the added anti-D serum, but this is

unlikely since the Mg^{2+} concentration would be of the order of 0.5 mM which is sufficient for cytolysis (Goldstein & Gomperts, 1975).

A similar phenomenon is seen in table 8.0.2 with effector cells from a different donor and a wider range of concentrations of chelating agent. Virtually complete inhibition is again seen at 2.0 mM with both EDTA and EGTA, and there is a dose-dependent inhibition below this concentration. Again, the EDTA/EGTA inhibition curves are very similar, suggesting that both Mg^{2+} and Ca^{2+} are required for lysis and that Mg^{2+} alone is insufficient.

These results are in disagreement with those of Goldstein & Smith (1976) who demonstrated the presence of a significant degree of lysis of sheep RBC in the absence of both cations.

Table 8.0.1 REQUIREMENT FOR DIVALENT CATIONS - I

	% specific lysis at concentration shown (mM)		
	nil	0.2	2.0
EDTA	63.2	25.7	1.8
EGTA	63.2	34.3	4.6

E/T 10:1 (4×10^4 RBC); 18hr. culture; adherent monocytes removed. Ca^{2+}/Mg^{2+} free TC 199 used but AB serum supplement contains Mg^{2+}/Ca^{2+}

Table 8.0.2 REQUIREMENT FOR DIVALENT CATIONS - II

	% specific lysis at concentration shown (mM)						
	nil	0.0625	0.125	0.25	0.1	1.0	2.0
EDTA	74.7	64.5	56.2	56.5	50.7	46.1	-0.9
EGTA	74.7	67.7	60.6	55.8	45.5	39.9	7.9

Legend as for table 8.0.1

9.0 EFFECTS OF IMMUNOGLOBULINS ON K-CELL LYSIS

9.1 Native immunoglobulins IgG, IgA, IgM

In the present system, it is known that IgG antibody was responsible for inducing K-cell lysis since only IgG anti-D was present in the serum and the IgG fraction of this serum was equally potent. The effects of purified immunoglobulins on the K-cell lysis of pre sensitised RBC are shown in table 9.1.1. It can be seen that up to a concentration of 30 µg/ml there is no inhibition by any of the immunoglobulin classes IgG, IgA or IgM.

The effects of higher concentrations of normal IgG are shown in table 9.2.1 and 9.2.2 where there is some inhibition seen at concentrations above 166 µg/ml and which is most noticeable at 1666 µg/ml (table 9.2.1; reduction from 37.1% S.L. to 0.5%). This suggests that at the highest concentrations there is competition by free IgG Fc portions with cell-bound IgG anti-D.

Although there is no inhibition with the concentrations of purified IgA and IgM used, these levels are lower than the expected levels of IgA and IgM in the serum supplements used in culture. Inhibition was therefore attempted by using crude myeloma sera with very high levels of paraprotein, and low contaminating levels of the other immunoglobulin classes. The results are shown in table 9.1.2 and 9.1.3.

Table 9.1.1 EFFECT OF INCUBATION WITH IgG, IgA, IgM ON K-CELL LYSIS

	Immunoglobulin concentration per culture		
	30 µg/ml	3 µg/ml	nil
IgG	70.9*	72.2	74.6
IgA	77.6	72.5	74.6
IgM	72.9	72.2	74.6

* % specific lysis in presence of immunoglobulin concentration as shown E/T 10:1 (4×10^4 0 R_1R_1 RBC per culture) adherent monocytes removed 18 hr. culture. RBC pre-sensitised with anti-D serum (Louden) neat

Table 9.1.2 EFFECT OF IgA AND IgM ON SPECIFIC LYSIS WITH FREE ANTI-D IN CULTURE

immunoglobulin per culture µg/ml	% specific lysis in presence of	
	IgA	IgM
nil	76.0	76.0
1	65.8	76.0
10	76.0	65.3
100	69.3	62.5
500	71.2	67.0
1000	72.0	72.3
10,000	76.7	72.2

E/T 10:1 (4×10^4 0 R_1R_1 RBC per culture); adherent monocytes removed; 18 hr. culture in presence of IgA or IgM at concentration shown.

Louden anti-D serum free in culture at 1/4

Table 9.1.3 EFFECT OF IgA AND IgM ON SPECIFIC LYSIS WITH PRESENSITISED RBC

	% specific lysis in presence of	
immunoglobulin concentration (μg/ml)	IgA	IgM
nil	29.7	29.7
10	29.9	28.0
100	29.6	30.3
500	25.1	30.1
1000	29.1	32.0
10,000	33.0	31.0

Legend as for table 9.1.2 except RBC pre-sensitised with Louden anti-D serum (neat)

In the experiments reported in table 9.1.2 free anti-D was present in culture, and this system is less sensitive to changes in culture conditions than the pre-sensitised RBC (see section I, para 7.0).

It can be seen that there is no significant inhibition with IgM or IgA even up to concentrations up to 10mg/ml (= 10 g/l) which is considerably higher than the concentrations of these immunoglobulins in normal plasma (1.0 g/l for IgA and 2.1 g/l for IgM). The actual concentration is higher than that shown since there will be some normal IgA and IgM percent in Louden serum added to the cultures. These results suggest that <u>in vivo</u> there will be no potential inhibition of specific lysis by IgA or IgM.

The results in table 9.1.3 show that even with the more "sensitive" system with pre-sensitised RBC, there is no significant inhibition up to a concentration of 10 mg/ml of IgA and IgM. This contrasts with the results obtained with native IgG, which completely inhibits at 1666 µg/ml with pre-sensitised RBC (table 9.2.1).

These results therefore indicate that the Fc receptors on effector K-cells are specific for IgG, and not for IgA or IgM, confirming the findings of Wisloff et al (1974b) who used a chicken RBC target.

Further investigations into the inhibition by IgG are reported under IgG subclasses (para 9.3).

9.2 Heat-aggregated IgG

9.2.1 Pre-incubation of effector cells with aggregated IgG

Aggregated IgG has altered Fc portions of the immunoglobulin molecule and binds more effectively to the Fc receptor of the mononuclear cells than does monomeric IgG (Dickler & Kunkel 1972). Pre-incubation of effector cells with aggregates should therefore be more effective in blocking K-cell lysis, assuming that an Fc receptor on the K-cell is required for lysis.

The results of such experiments are shown in figure 9.2.1 where there is complete inhibition of K-cell lysis on pre-incubation of effector cells with 5000 µg/ml aggregated IgG but only a reduction from 23.7 to 16% S.L. at the equivalent concentration of monomeric IgG. These cultures were performed in the absence of human serum containing any immunoglobulins apart from that in the cultures. The inhibition with non-aggregated IgG at the highest concentration used suggests that there is a degree of spontaneous aggregation.

9.2.2 Aggregated IgG added to cultures

The results to two separate series of experiments are shown in tables 9.2.1 and 9.2.2. In table 9.2.1 where aggregated IgG is compared with an equivalent concentration of non-aggregated IgG in culture, it can be seen that aggregated IgG is more efficient in inhibiting specific lysis at 1.6 µg/ml and 16.6 µg/ml of IgG but that there is little difference between them at 166 and 1666 µg/ml where there is complete inhibition with both materials. This suggests that, as above, the

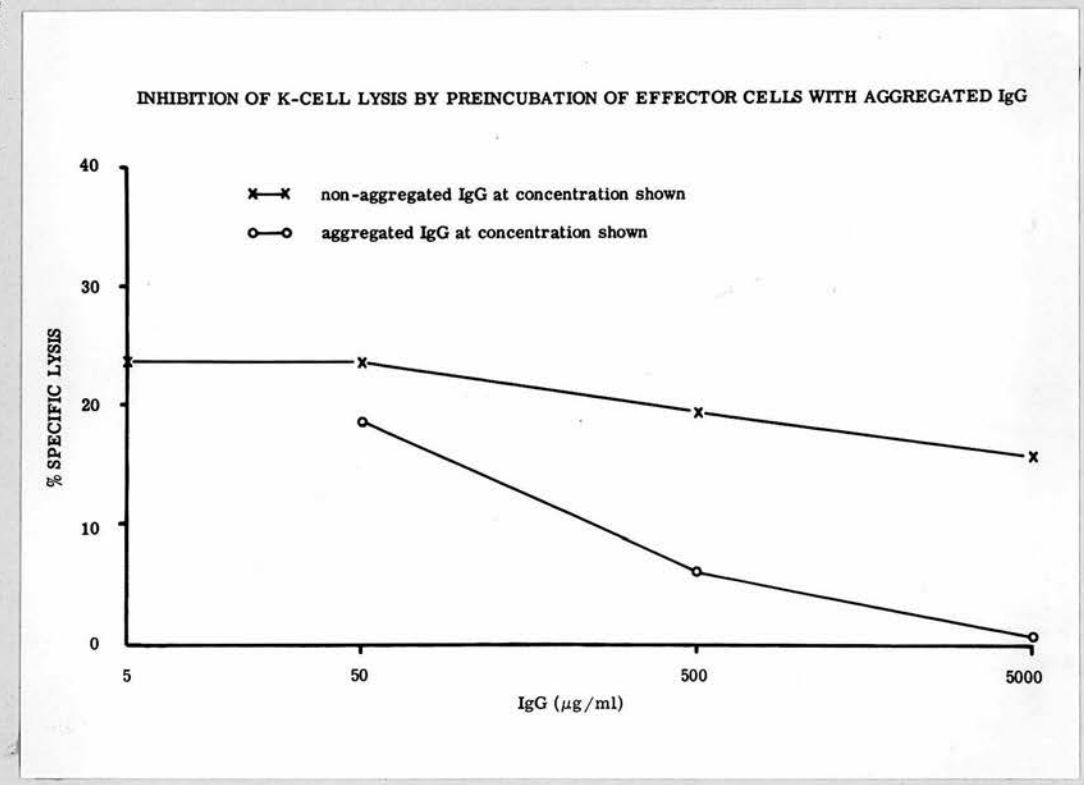

Fig. 9.2.1

E/T 10:1 (4 x10^4 RBC per culture) adherent monocytes removed 18 hr. incubation for K-cell lysis

preincubation with aggregated IgG as described in Chapt. III

Table 9.2.1 EFFECT OF AGGREGATED IgG IN CULTURE - I

IgG per culture (μg/ml)	% specific lysis with	
	aggregated IgG	non-aggregated IgG
nil	37.1	37.1
1.6	15.3	40.1
16.6	17.1	32.8
166	20.5	16.3
1666	0.6	0.5

E/T 10:1 (4×10^4 O R_1R_1 RBC per culture) pre-sensitised with Louden anti-D serum (neat) adherent monocytes removed.

18 hr. culture in presence of IgG as shown.

Table 9.2.2 EFFECT OF AGGREGATED IgG IN CULTURE - II

IgG per culture (μg/ml	% specific lysis with		
	aggregated IgG	non-aggregated IgG	ultracentrifuged* IgG
nil	28.5	28.5	28.5
5	23.0	19.1	22.9
50	24.2	22.4	19.3
500	9.5	14.2	18.6

conditions as for I but different donors

* non-aggregated material centrifuged to remove "spontaneous" aggregates

non-aggregated IgG has in fact spontaneously aggregated on storage.

Confirmatory evidence for this was obtained from the results shown in table 9.2.2 where a portion of the unaggregated IgG was ultra-centrifuged at 40,000 g to remove spontaneously-formed aggregates and the IgG then used immediately for inhibition assays. With this particular K-cell donor, inhibition was only seen at the highest concentration of 500 µg/ml where the percent S.L. has been reduced from 28.5% to 9.5% with aggregated IgG, to 14.2% with non-aggregated IgG and only to 18.6% with ultracentrifuged non-aggregated IgG.

9.3 IgG subclasses (IgG_1, IgG_2, IgG_3 and IgG_4)

It has been shown that the IgG subclasses which best induce K-cell lysis are IgG_1 and IgG_3 (see chapter I) and that these are the same subclasses which fix complement (Abramson & Schur 1972) but inhibition of IgG_1 or IgG_3 mediated lysis can be achieved with either IgG_1 or IgG_3 ie. there is a degree of identity between the Fc receptors (Holm et al 1974).

Since partial inhibition was demonstrated in the above experiments and good inhibition with aggregated IgG, this phenomenon was investigated further by the use of IgG subclasses.

9.3.1 Native IgG subclasses

The effects of the four IgG subclasses on RBC lysis are shown in tables 9.3.1/1 and 9.3.1/2 where a comparison has been made between anti-D pre-sensitised RBC and free anti-D in culture.

In table 9.3.1/1 it can be seen that there is approximately 25% inhibition with IgG_1 and IgG_2 at the highest concentrations of 1666 µg/ml and negligible inhibition at all concentrations with IgG_3 and IgG_4.

With anti-D present throughout culture (table 9.3.1/2) the results are more variable, and no definite pattern of inhibition is seen with any of the IgG subclasses. At the highest concentration of 1250 µg/ml there is apparently 18.3% inhibition with IgG_4, but insignificant changes with the other subclasses. It

Table 9.3.1/1 EFFECT OF NATIVE IgG SUBCLASSES - PRESENSITISED RBC IN CULTURE

concentration of immunoglobulin µg/ml	% specific lysis with							
	IgG_1		IgG_2		IgG_3		IgG_4	
	% S.L.*	% I**	% S.L.	% I	% S.L.	% I	% S.L.	% I
nil	22.9	-	22.9	-	22.9	-	29.7***	-
16.6	24.4	0	26.5	0	26.1	0	32.1	0
166	25.4	0	20.7	9.6	23.0	0	34.7	0
833	22.8	0.4	23.0	0	23.2	0	34.7	0
1666	17.3	24.5	17.3	24.5	21.3	7.0	28.0	5.7

* % specific lysis at E/T 10:1 (4×10^4 RBC per culture); adherent monocytes removed; 18 hr. culture in presence of IgG subclass concentration as shown

** % inhibition compared with nil IgG as 100%

*** results obtained with different donor hence different nil result

Table 9.3.1/2 EFFECT OF NATIVE IgG SUBCLASSES FREE ANTI-D IN CULTURE

concentration of immunoglobulin µg/ml	% specific lysis with							
	IgG_1		IgG_2		IgG_3		IgG_4	
	% S.L.	% I	% S.L.	% I	% S.L.	% I	% S.L.	% I
nil	81.5	-	81.5	-	81.5	-	81.5	-
12.5	67.8	16.8	80.9	0.7	79.2	2.8	75.1	7.9
125	64.2	21.2	-	-	74.4	8.7	71.5	12.3
625	77.2	5.3	82.8	0	61.2	24.9	83.4	0
1250	76.1	6.6	76.1	6.6	100	0	66.6	18.3

legend as for table 9.3.1/1 except that Louden anti-D added directly to cultures (1/4)

is likely that the uninhibited control cultures in this experiment gave results, by chance, above the average and that there is in fact no significant pattern of inhibition with any of the subclasses. Comparison of the above results with those for IgA and IgM suggest that inhibition is only seen at high concentrations in the more sensitive assay with anti-D coated RBC.

9.3.2 Heat-aggregated IgG subclasses

In these experiments the IgG subclasses were subjected to exactly the same aggregation treatment although it is known that the degree of aggregation produced is not necessarily the same for each subclass (MacLennan et al 1973). Similar results to those found by MacLennan et al (1973) were found and are shown in table 9.3.2. As with pooled normal IgG, efficient inhibition of lysis is seen with concentrations as low as 16.6 µg/ml of subclass IgG (compare table 9.2.1). With IgG_1 there is 84.7% inhibition at 16.6 µg/ml and complete inhibition at 166 µg/ml and above. With IgG_2 and IgG_4 there is weak and inconsistent inhibition, and with IgG_3 there is a dose-dependent inhibition of specific lysis from 31.4% inhibition at 16.6 µg/ml to 68.1% at 1666 µg/ml.

These results therefore suggest that the aggregated subclasses IgG_1 and IgG_3 are the most efficient at inhibiting specific lysis in contrast to the native subclasses where IgG_1 and IgG_2 are marginally effective at the concentrations tested.

Table 9.3.2 EFFECT OF AGGREGATED IgG SUBCLASSES

concentration of immunoglobulin µg/ml	% specific lysis with							
	IgG_1		IgG_2		IgG_3		IgG_4	
	% S.L.*	% I**	% S.L.	% I	% S.L.	% I	% S.L.	% I
nil	22.9	-	22.9	-	22.9	-	22.9	-
16.6	3.5	84.7	22.9	0	15.7	31.4	21.1	7.9
166	0.8	100	22.3	2.6	14.7	35.8	22.1	3.5
833	-0.7	100	20.9	8.7	12.1	47.2	23.2	0
1666	-0.6	100	20.8	9.2	7.3	68.1	20.0	12.7

* % specific lysis at E/T 10:1 (4×10^4 RBC per culture) adherent monocytes removed. 18 hr. culture in presence of IgG subclass concentration as shown

** % inhibition compared with no IgG as 100%

9.4 Conclusions

Of the three major immunoglobulin classes tested, IgA and IgM appear ineffective at any of the concentrations tested up to a maximum of 10,000 µg/ml (= 10 g/l) which is well above the normal mean serum concentrations of these immunoglobulins (1.0 g/l for IgM and 2.1 g/l for IgA). Pooled native IgG is inhibitory above concentrations of 1666 µg/ml, and at lower concentrations of heat aggregated material. These results are in keeping with published observations that the Fc-receptors on the effector K-cells are specific for IgG (see chapt. I).

The results obtained with IgG subclasses are more difficult to interpret, since it is known that the sensitising antibody is almost entirely of IgG_1 with some IgG_2 (see chapter III, section 5.0) and the minor inhibition seen with unaggregated IgG subclasses would suggest that the IgG_1 and IgG_2 subclasses are more effective at inhibiting specific lysis. With aggregated IgG subclasses however, IgG_1 and IgG_3 are unequivocally more efficient inhibitors, although IgG_1 is better than IgG_3. The most efficient inhibition is therefore seen with IgG of the same subclass as the inducing antibody. It is probably that there is cross-reactivity between IgG_1 and IgG_3 in blocking the K-cell Fc receptor for activated IgG_1 on the RBC. These observations are in agreement with those of Holm et al (1974) and Larsson et al (1975). On the other hand, it may be that IgG_1 and IgG_3 aggregate more efficiently than IgG_2 and IgG_4 under the conditions used and one is merely observing the more efficient inhibition of aggregated IgG over native IgG. However, the

aggregating conditions were the same as those of MacLennan et al (1973), who showed that IgG_3 aggregated best, IgG_1 least, and IgG_2 and IgG_4 were intermediate. Overall, the above results indicate the more efficient inhibition of IgG_1 inducing-antibodies by IgG of the same subclass, but IgG_3 and IgG_2 are also able to inhibit partially. The Fc-receptor on the K-cell must therefore be reactive with all three IgG subclasses but not with IgA or IgM as has been suggested previously (see introduction, table 2.3.1).

SECTION III - INVESTIGATION INTO THE NATURE OF THE EFFECTOR CELL

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1.0 INTRODUCTION

It has already been demonstrated in previous experiments that monocytes can be reduced from approximately 15% to 2.5% by plastic dish adherence (section I, table 5.1) and to approximately 2% by nylon columns (section I, table 6.1) and that the dose-response curves obtained by altering the E/T at fixed anti-D concentration with and without monocyte removal are very similar (section I, figure 5.2.1).

It has also been shown that the dose-response curves obtained by diluting anti-D at a fixed E/T of 10:1 with and without monocyte removal are very similar and that there is usually an increase in specific lysis at a given anti-D concentration following monocyte removal (section I, figure 6.2.1 and 6.2.2).

Phagocytosis does not play a significant role in specific lysis (section II, table 3.0.2) and the results obtained with metabolic inhibitors indicate that as the numbers of monocytes are reduced a higher concentration of inhibitor is required to achieve the same degree of inhibition (section II, para 7.0). With cytochalasin B, there is a significant increase of lysis at low concentrations suggesting that there may be lysis as a result of release of lysosomal enzymes from monocytes under these conditions.

The specific lysis as described above therefore appears to be due to other than monocytes as described

by conventional criteria. The role of monocytes was investigated more formally in the present series of experiments where the depletion of monocytes was examined in more detail. Various manipulations were done on the effector cell populations, to attempt identification of the cell surface markers.

2.0 EFFECTS OF REMOVAL OF MONOCYTES BY NYLON WOOL COLUMNS

2.1 Efficiency of removal of monocytes

As seen in section I, table 3.2 the recovery of mononuclear cells following nylon wool column passage is reproducible for a given individual (coefficient of variation 14.8%). The overall results obtained in 61 experiments are shown in table 2.1.1 where the average recovery is 53.9%. There is thus a considerable retention of cells in the nylon columns, some of which must be non-specific since at the most, 20-25% of the original cell population are identifiable as monocytes. The efficiency of removal of monocytes is indicated by the results in table 2.1.2 where three methods of identification have been used. Further detailed comparison between the three methods in individual experiments can be obtained from table 2.3.1.

From table 2.1.2 it is apparent that the methods using living cell suspensions ie. phase contrast and toluidine blue supervital staining, identify significantly less monocytes than does the less subjective non-specific esterase method. It can also be seen that monocytes in the post-nylon suspensions are over-estimated by the two former methods. The difference in estimation of monocyte numbers probably lies in the subjective criteria of size and morphology used for identification in that 1) a number of monocytes may have the morphology of small lymphocytes (5-10 microns diameter) which are identified only histochemically,

Table 2.1.1 NYLON WOOL COLUMN PASSAGE - RECOVERY OF MONONUCLEAR CELLS

N	61
mean	53.9
S.D.	16.2
range	21.8 - 89.0

Table 2.1.2 REMOVAL OF MONOCYTES BY NYLON WOOL COLUMNS

Estimated by :		% monocytes	
		pre nylon	post nylon
phase contrast :	mean	12.49	0.85
	S.D.	3.89	0.78
	range	7 - 23	0 - 4
Toluidine Blue :	mean	14.2	1.0
	S.D.	4.15	0.87
	range	9 - 22	0 - 3
Non-specific :	mean	20.68	0.38
	S.D.	5.13	0.59
Esterase	range	12 - 36	0 - 15

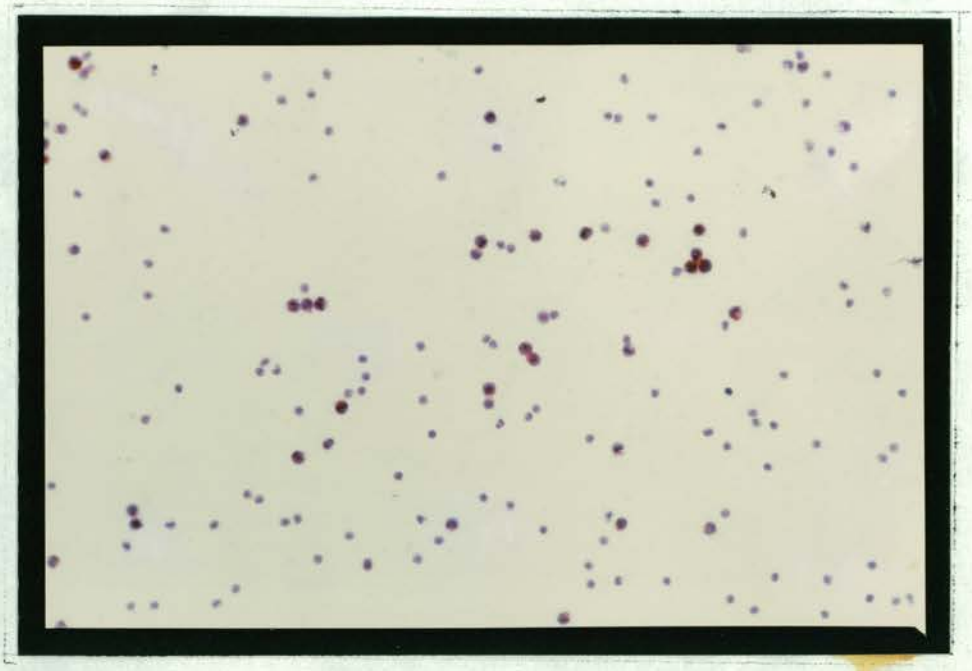


Fig. 2.1.1 Non-specific Esterase stain - pre-nylon (x 50 magnification)

Note: monocytes with red cytoplasm

(see also fig 2.5, p. 46)

Fig. 2.1.2 Non-specific Esterase stain - post nylon (x 100 magnification)

Note: absence of monocytes

and 2) a number of large lymphocytes (>10 microns diameter) may be misclassified as monocytes whereas they are esterase negative. Examples of esterase staining, pre and post nylon column, are shown in figs. 2.1.1 and 2.1.2.

2.2 Effects on lymphocyte subpopulations

The effects of nylon column passage on lymphoid cell subpopulations as determined by surface marker methods are shown in table 2.2. The only significant changes (apart from removal of monocytes) is a relative enrichment of T cells and depletion of surface-immunoglobulin-bearing B cells after nylon passage. This is to be expected since it is known that SIg. bearing B cells will bind to nylon wool in the presence of serum and this property can be used to remove such cells from suspensions (Werner et al 1976). It is interesting that cells bearing C3 and Fc markers do not necessarily segregate with SIg. bearing cells and it is likely that different but overlapping subpopulations of lymphoid cells bear the different markers. This has been noted previously (Jondal et al 1973).

The recovery of cells from the nylon columns was technically unsatisfactory and in only a few instances were sufficient cells obtained for surface marker studies (table 2.2). Little can be deduced from the small numbers except that the T cells are depleted as expected, and that there are relatively few Fc bearing cells in the recovered cell suspensions. The cell population

Table 2.2 EFFECT OF NYLON WOOL COLUMN ON LYMPHOID CELL POPULATIONS

	PRE-NYLON PASSAGE (%)				POST-NYLON PASSAGE (%)				COLUMN RECOVERED (%)			
	AETr.* (T)	S.Ig. (B)	EACr. (C3)	EAr. (Fc)	AETr. (T)	S.Ig. (B)	EARr. (C3)	EAr. (Fc)	AETr. (T)	S.Ig. (B)	EACr. (C3)	EAr. (Fc)
1	81.0	-**	11.1	6.3	86.2	-	8.8	11.2	-	-	-	-
2	75.5	-	21.5	17.3	83.0	-	18.8	16.8	-	-	-	-
3	82.3	10.3	-	-	84.0	7.0	-	-	-	-	-	-
4	87	7.0	-	-	92.5	4.8	-	-	-	-	-	-
5	59.4	7.8	8.0	7.4	73.3	2.8	4.0	5.8	-	-	-	-
6	82.0	4.9	14.3	-	85.5	1.0	9.8	-	25.3	-	5.0	2.0
7	50.5	8.0	-	-	71.5	2.5	-	-	22.5	-	5.0	1.3
8	-	-	25.3	10.5	-	-	30.8	12.5	-	-	-	-
Mean	70.4	7.6	16.0	9.4	82.3	3.6	14.4	11.3	23.9		7.8	1.7
S.D.	13.7	2.0	7.2	5.9	7.4	2.3	10.6	4.5	2.0	-	3.9	0.5
N	7	5	5	4	7	5	5	4	2		2	2

* AETr = AET-sheep RBC rosettes; S.Ig. = Surface immunoglobulin by immunofluorescence
EACr = complement C3 sheep RBC rosettes ; EAr. = IgG Fc sheep RBC rosettes

** not tested

paired t test pre vs. post nylon:

AETr	p <0.01	>0.005
SIg	p <0.005	>0.0025
EACr	p <0.25	>0.20
EAr	p <0.4	>0.35

bearing Fc receptors also contains the effector K-cell population and it is interesting that the % S.L. in the column recovered cells is also very low (table 2.3.2).

2.3 Effect on cytotoxic activity

The results from a series of experiments where pre- and post nylon K-cell activity was correlated with the number of monocytes are shown in table 2.3.1. It can be seen that despite the removal of monocytes to undetectable levels in many cases, there is no loss of specific lysis and in fact there is a statistically significant increase in specific lysis (mean of 32.8% S.L. pre to 39.7% S.L. post nylon ; $p < 0.05 > 0.025$ paired t test). This suggests that there is a relative enrichment of K-cells following monocyte depletion in cell suspensions. Correlation tests (regression coefficients) showed that there was no significant correlation between specific lysis and the % monocytes estimated by any of the methods used either pre or post nylon passage.

In a small series of experiments it was possible to obtain sufficient material for correlation of monocytes with specific lysis on cells recovered from the nylon columns as well as pre and post nylon passage (table 2.3.2). The results obtained confirm the previous findings, but the increase in post nylon specific lysis was not statistically significantly higher in view of the smaller numbers (mean 36.3% S.L.

Table 2.3.1 REMOVAL OF MONOCYTES ON NYLON WOOL - EFFECT ON SPECIFIC LYSIS AND NON-CORRELATION WITH NUMBER OF MONOCYTES

	anti-D source	pre nylon				post nylon			
		% monocytes			% S.L.	% monocytes*			% S.L.
		PC	TB	Est.		PC	TB	Est.	
1.	a	23	-**	-	46.1	<17	-	-	50.0
2.	b	20	-	-	24.1	5	-	-	35.1
3.	b	15	-	-	47.0	1	-	-	65.8
4.	b	9	-	-	7.4	1	-	-	34.3
5.	b	13	-	-	50.9	3	-	-	62.3
6.	b	15	-	-	45.3	4	-	-	35.3
7.	b	16	-	-	21.8	0	-	-	40.7
8.	b	10	-	-	17.4	1	-	-	12.1
9.	b	7	-	-	45.8	<1	-	-	54.5
10.	b	8	-	-	9.6	1	-	-	20.8
11.	b	17.5	-	-	7.2	<1	-	-	20.8
12.	b	10	14	-	73.7	0	-	-	74.1
13.	b	10	12	12	8.9	0	0	0	10.8
14.	b	9	15	15	65.5	0	1	0	59.9
15.	a	7	10	19	77.7	0	1	1.5	46.6
16.	a	8	9	20	11.5	1	2	0	11.4
17.	a	16	16	16	4.2	1	2	0	16.4
18.	a	14	-	12	41.7	<1	-	0	43.7
19.	a	20	-	22	38.8	1	-	0.3	52.9
20.	a	22	-	19	50.3	<1	-	0	69.8
21.	c	12	-	31	13.1	<1	-	0	43.0
22.	c	8	15	12	14.3	<1	1	0	13.9
	mean	13.16	13.0	17.8	32.8	1.02	1.17	0.18	39.7
	SD	5.04	2.71	5.88	22.82	1.30	0.75	0.47	20.30
	SEM	1.1	1.0	1.9	4.9	0.3	0.3	0.2	4.3
	N	22	7	10	22	22	6	10	22

* P.C. = phase contrast; T.B. = Toluidine Blue; Est. = non-specific esterase determinations.

** not tested. * value of 0.5% used for calculations.

O R_1R_1 target RBC used for all cultures (4×10^4 per culture) E/T ratio 10:1. 18 hr. culture.

a = Louden serum 1/3 free in culture; b = Louden IgG II pre-sensitised RBC; c = Louden IgG I pre-sensitised RBC

Difference between % S.L. pre and post-nylon is statistically significant (paired t test) $p < 0.05 > 0.025$

Table 2.3.2 EFFECT OF REMOVAL OF ESTERASE POSITIVE MONOCYTES ON K-CELL LYSIS - NYLON COLUMN PASSAGE

	anti-D source	Pre-nylon		Post-nylon		Nylon recovered	
		% Est. +ve	% S.L.	% Est. +ve	%S.L.	% Est. +ve	% S.L.
1.	Louden 1/3 free	12	41.7	0	41.7	18	10.1
2.	Louden 1/3 free	22	38.8	0.3	52.9	26	9.5
3.	Louden 1/3 free	19	50.3	0	69.8	18	24.2
4.	IgG I presens.	12	14.3	0	13.9	26	8.0
	mean	16.25	36.28	0.08	45.08	22.0	12.95
	SD	5.06	15.44	0.15	23.4	4.62	7.55

* E/T ratio 10:1 (4 x 10^4 0 R_1R_1 RBC per culture)

correlation coefficients for % monocytes vs. % specific lysis

pre-nylon r = 0.04 : not significant

post-nylon r = 0.22 : not significant

nylon recovered r = 0.64 : not significant

paired t test for % specific lysis

pre vs. post % S.L. $p < 0.10 > 0.05$

pre vs. column % S.L. $p < 0.025 > 0.0125$

post vs. column % S.L. $p < 0.025 > 0.0125$

pre nylon to 45% S.L. post nylon). The specific lysis with column-recovered cells was significantly lower than the pre- or post nylon passaged cells despite having higher numbers of monocytes and this is confirmatory evidence that the monocytes are not the active lytic cell in this system.

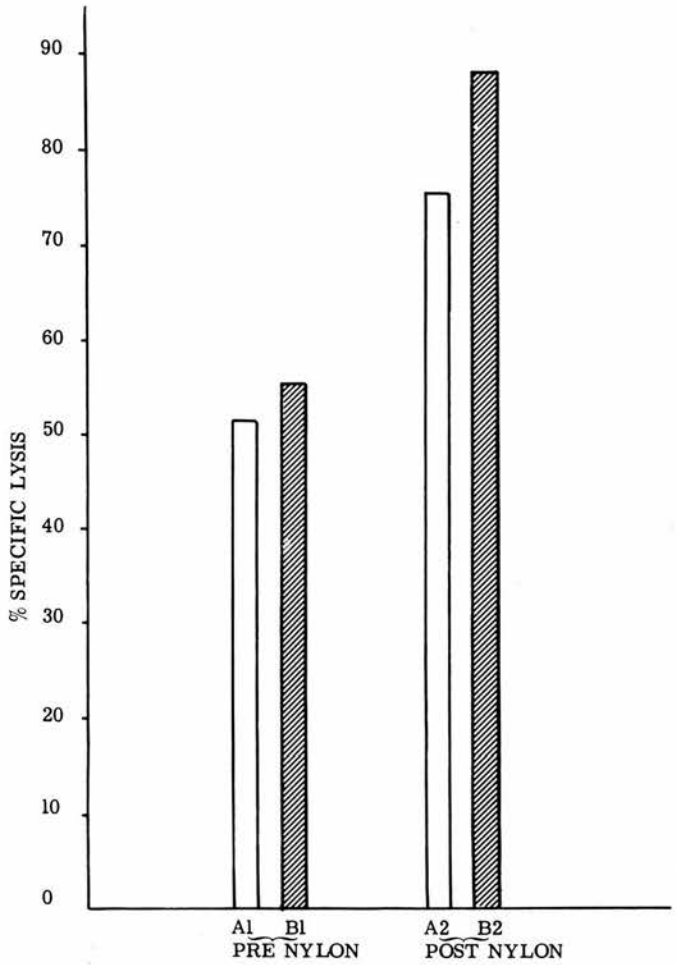
3.0 REMOVAL OF MONOCYTES BY CARBONYL IRON PHAGOCYTOSIS

The effect of removing phagocytic monocytes as well as nylon adherent monocytes is shown in fig. 3.0. It can be seen that carbonyl iron treatment alone reduces the percent monocytes from 14.4% to 0.7% and that the % specific lysis increases simultaneously from 51.5% to 55.5%. Nylon wool alone reduced the monocytes from 14.4% to 1.6% and the % specific lysis increased to 75.5%. Combined nylon and carbonyl iron treatment reduced monocytes to undetectable levels and a further increase in % specific lysis is seen to 88.2%.

This indicates that the lysis of target cells does not correlate with the number of monocytes (there is in fact an inverse correlation) thus confirming earlier observations with nylon wool passage that the removal of monocytes may actually concentrate an unidentified effector cell present in the mononuclear cell suspension.

There is very good correlation between the reduction in monocytes obtained on the basis of adherence and of phagocytosis indicating that nylon wool removal of monocytes (as in all other experiments) is a valid method.

EFFECT OF REMOVING MONOCYES BY NYLON WOOL
ADHERENCE AND CARBONYL IRON PHAGOCYTOSIS



A No Carbonyl Iron treatment

B Carbonyl Iron treatment

A1 14.4% monocytes
A2 1.6% monocytes

B1 0.7% monocytes
B2 0% monocytes

E/T 10.1
Lym donor Brr
Louden serum anti-D 1/3 free
18 hr. culture

Fig. 3.0

4.0 EFFECT OF DEPLETING T-LYMPHOCYTES BY E-AET ROSETTE SEDIMENTATION

It was shown in table 2.2 that there was a significant increase in percent T cells following nylon column passage and at the same time, an increase in specific lysis. Although other authors' experimental systems conclusively demonstrate that the T cell is not involved in K-cell lysis (chapt. I, table 2.2.4), this cannot be formally excluded on the basis of the above results, and indeed one might conclude that there was good statistical correlation between percent T cells and % specific lysis Experiments were therefore devised to investigate whether or not the T cells are involved in red cell lysis and this involved the depletion of cell suspensions of T lymphocytes by E-AET rosetting sedimentation (see methods).

The effects of the E-AET rosette depletion are shown in figs. 4.0.1 and 4.0.2. It can be seen that at low E/T ratios where the system is more "sensitive", the specific lysis is much lower with the T-enriched population than the T-depleted population (1% E-AET rosettes, where the % S.L. is in fact greater than that shown by the unseparated original cell suspension (52% E-AET rosettes). This suggests that non-T cells are involved which are selectively "concentrated" after removal of the T lymphocytes.

With both donors A and B, the differences between T-depleted and enriched populations were not seen at the higher E/T ratios (5:1 and 10:1 respectively). It is likely that the relatively incomplete separation of T lymphocytes

Fig. 4.0.1

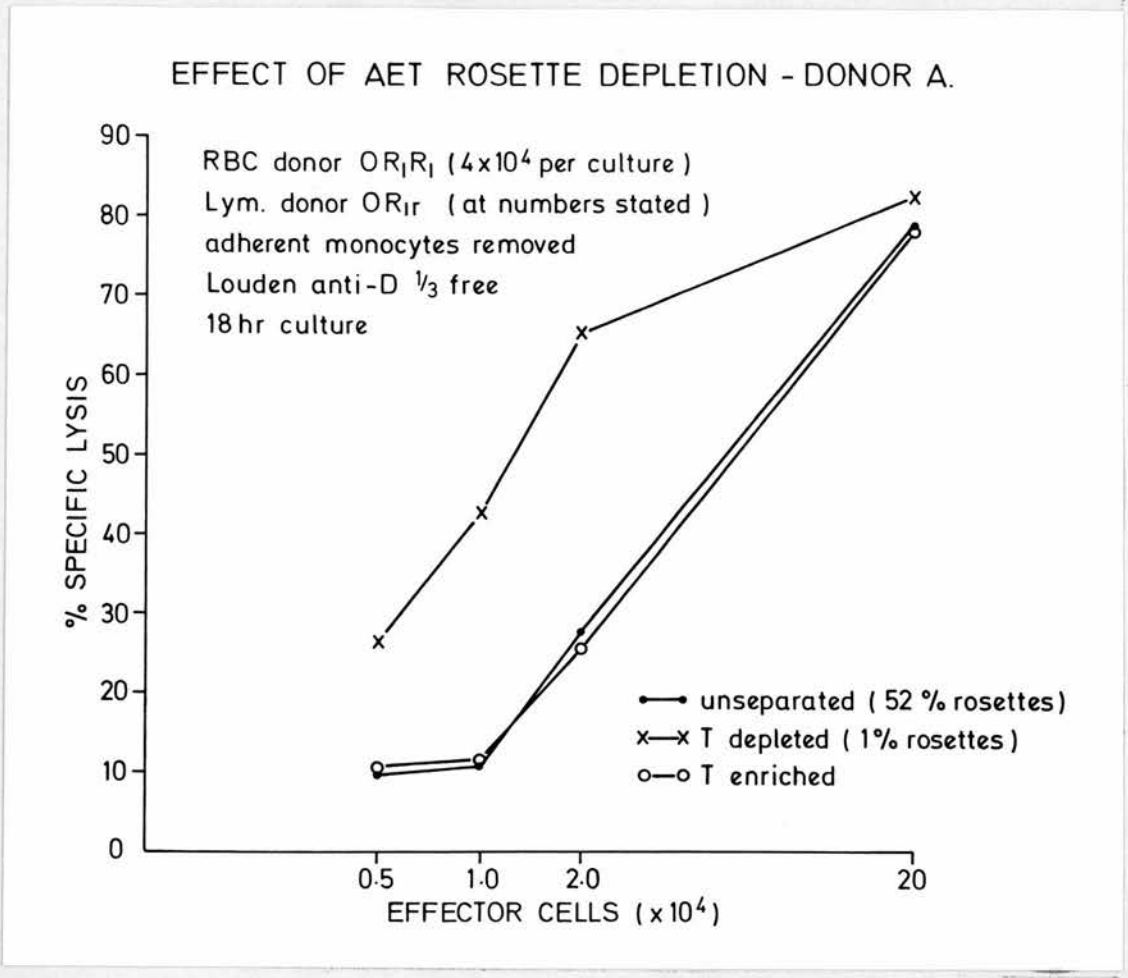
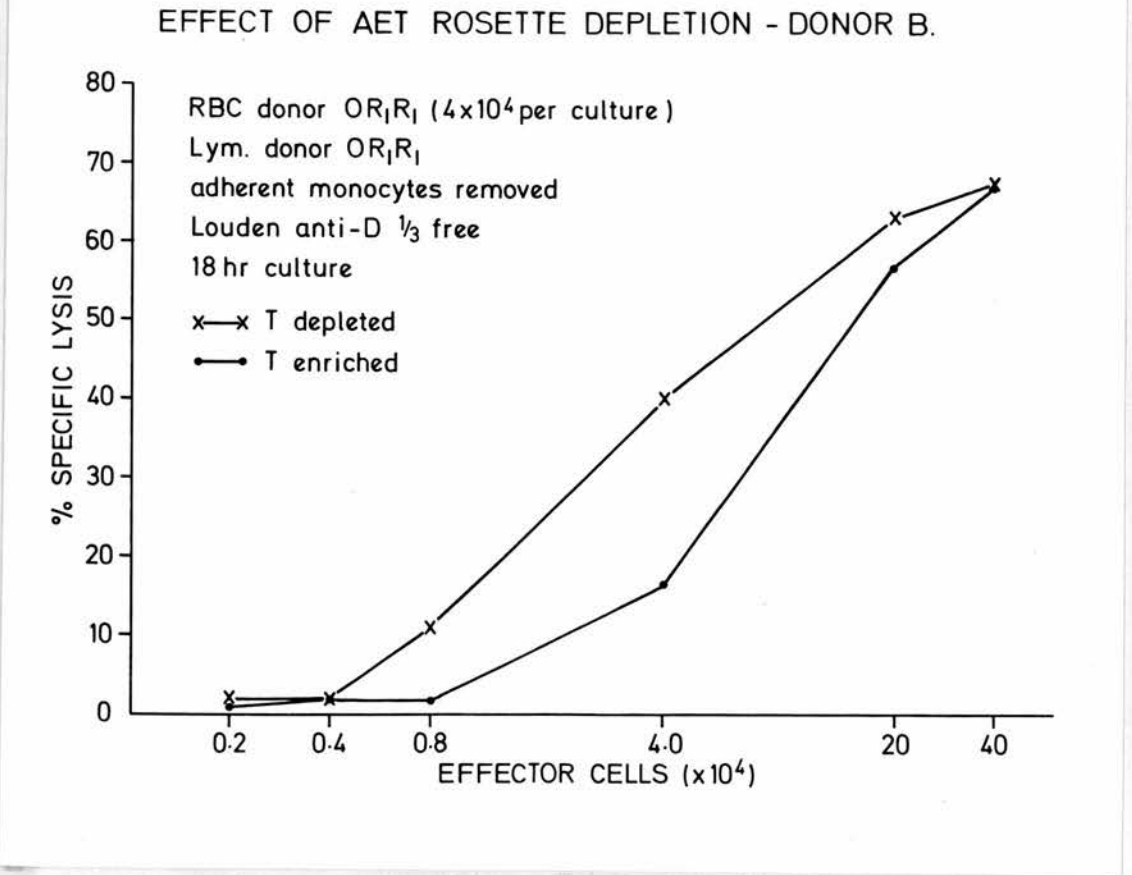


Fig. 4.0.2



results in contamination of the T-enriched fraction with K-cells and that these cells are very efficient in producing lysis. Both donors have very good K-cell function in that 70-80% S.L. is seen (at the level of the dose-response plateau- section I, fig. 5.2.2). The contamination of K-cells in the T-enriched population eventually results in the same maximum % specific lysis as the untreated original suspensions.

However, it seems reasonable to conclude that the numbers of T lymphocytes do not correlate with K-cell function and that, in common with other published work, the T lymphocyte is not involved in the ADCC lysis of red cells.

5.0 EFFECT OF DEPLETED C3 RECEPTOR BEARING CELLS

It was shown in table 2.2 that there was no significant alteration in percent EAC3 rosette-forming lymphoid cells following nylon column passage. The presence of the C3 receptors on the human K-cell has been demonstrated by several workers (chapt. I, table 2.2.5) and therefore experiments were performed to assess the association of C3 receptor bearing cells and K-cell lysis.

5.1 By EAC3 rosette sedimentation

The depletion of cell-suspensions of C3 bearing-cells was achieved by sedimentation of EAC3 rosetting cell suspensions (see methods). Results of experiments on three donors are shown in fig. 5.1.1, 5.1.2 and 5.1.3, and are essentially inconclusive. Only in donor A was good separation achieved and in this case the C3 enriched population had lower cytotoxic activity than the depleted population (fig. 5.1.1), suggesting that the C3 receptor is not necessarily present on the K-cell. With donors B and C there was no difference between the depleted or enriched populations, and in any case the % specific lysis seen with the treated suspensions was much lower than that with the untreated origianal cell suspension. This suggests that the methodology is in some way at fault and interfering with K-cell function.

5.2 By EAC3 monolayers

Preliminary experiments showed that incubation of

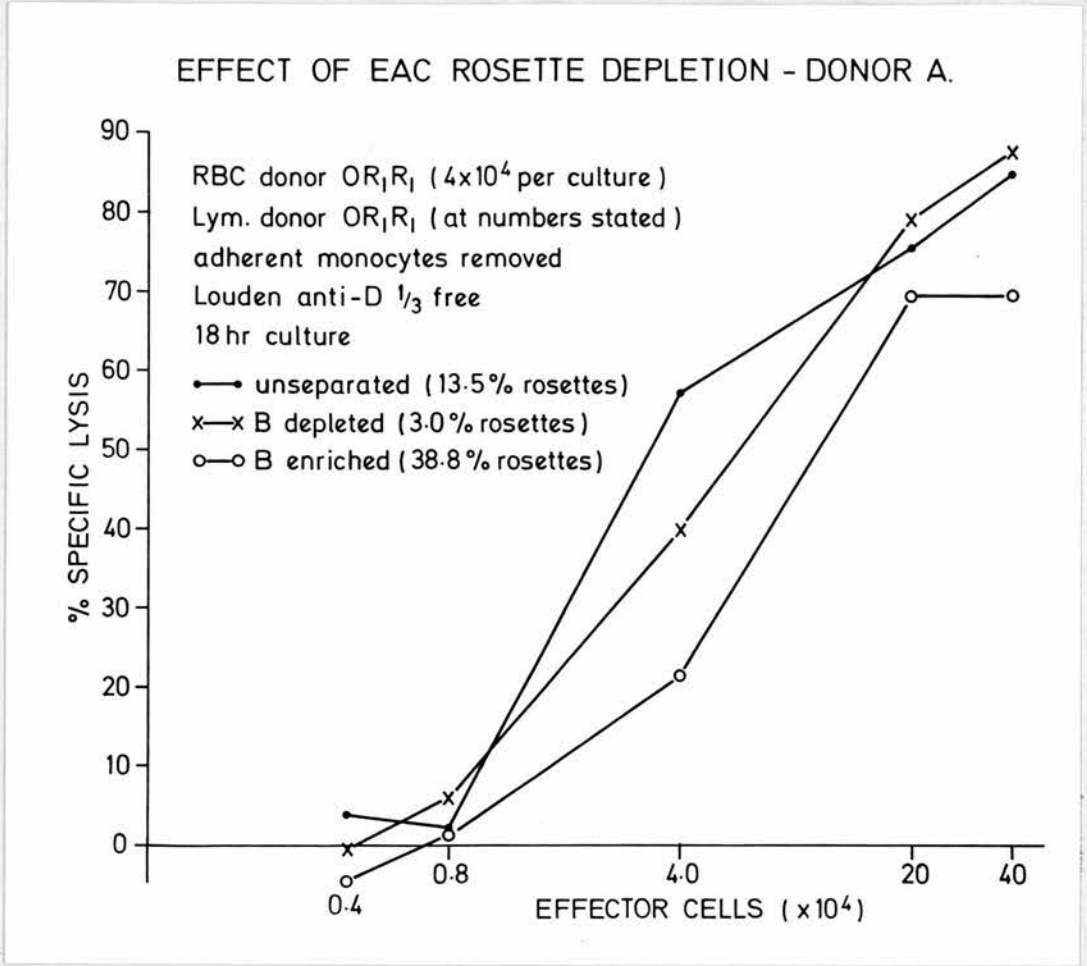


Fig. 5.1.1

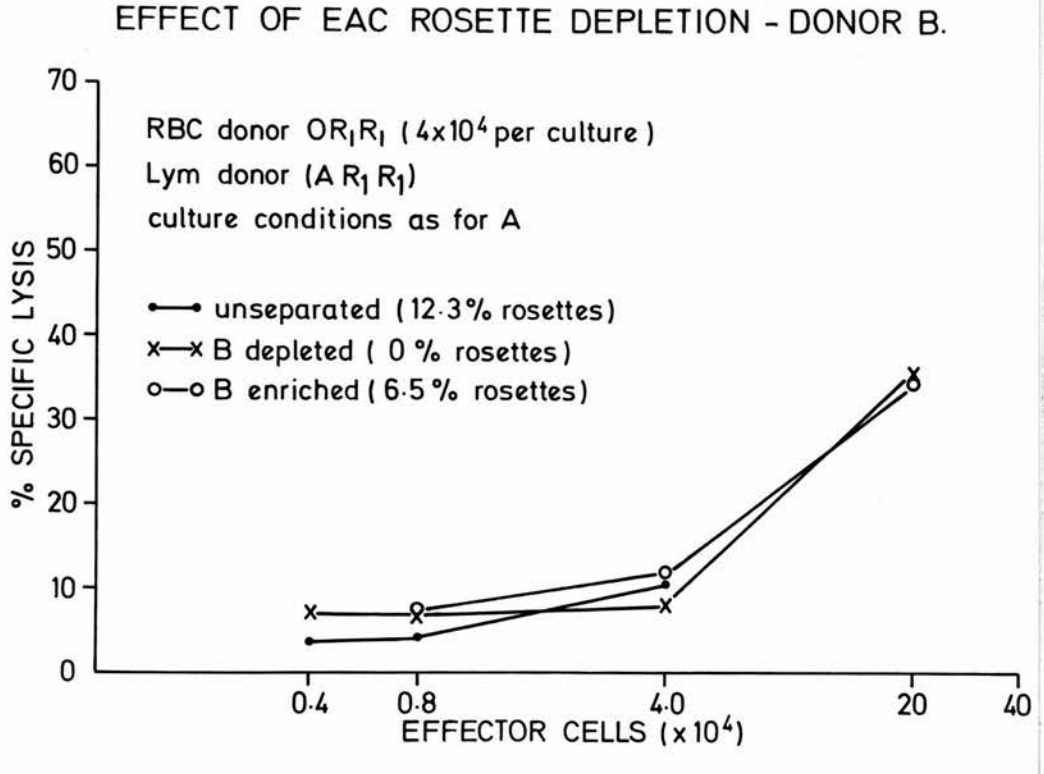


Fig. 5.1.2

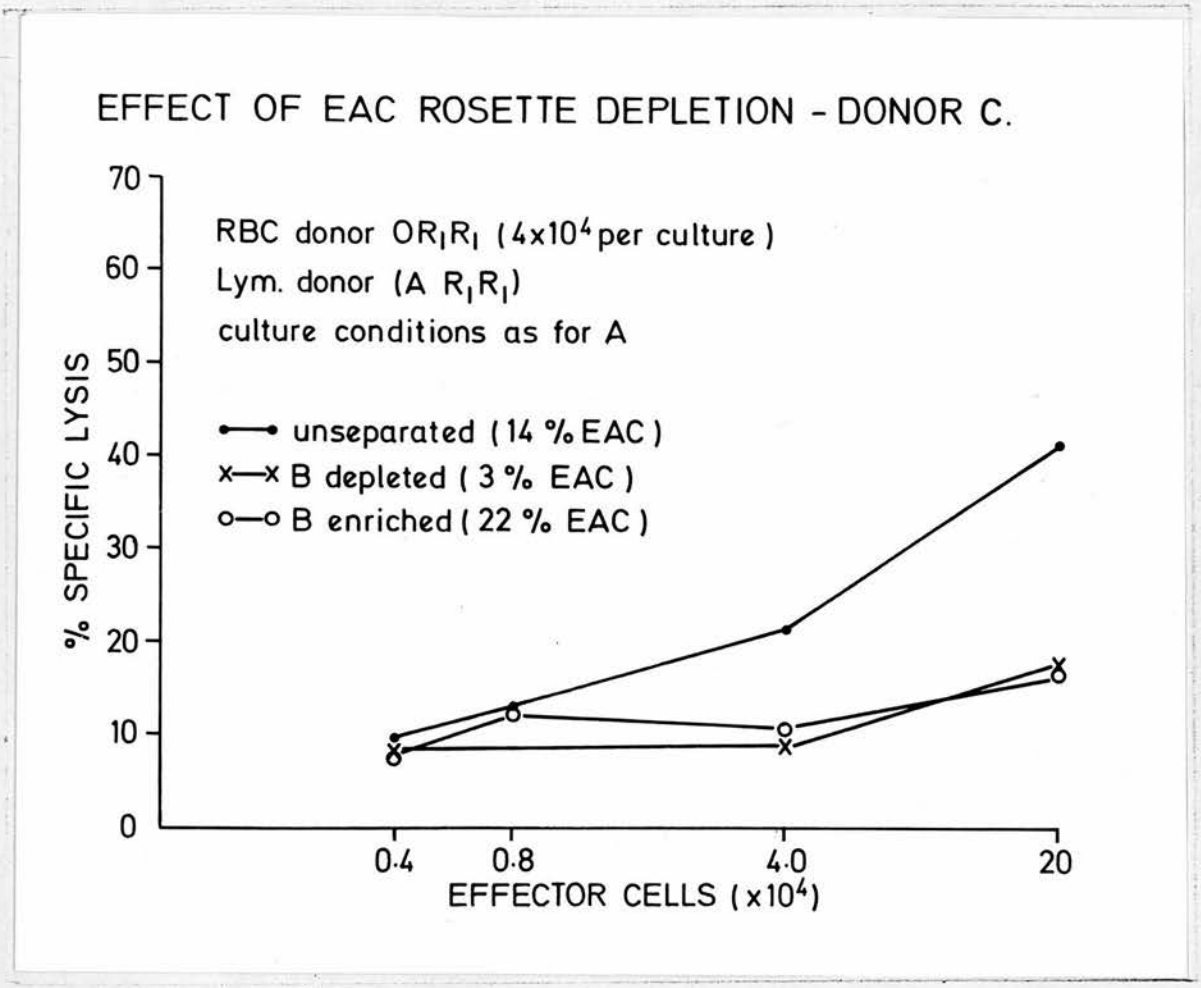

EFFECT OF EAC ROSETTE DEPLETION - DONOR C.
RBC donor OR1R1 (4x10⁴ per culture)
Lym. donor (A R1R1)
culture conditions as for A
unseparated (14 % EAC)
B depleted (3 % EAC)
B enriched (22 % EAC)
% SPECIFIC LYSIS
70
60
50
40
30
20
10
0
0·4
0·8
4·0
20
EFFECTOR CELLS (x10⁴)

Fig. 5.1.3

the effector population on plain sheep RBC monolayers, under the same conditions as for EAC3 monolayers, had no effect on specific lysis (47% compared with 42% S.L.). The effects of depleting C3 receptor bearing cells by EAC3 monolayers are shown in tables 5.2.1 and 5.2.2. It can be seen that the results are again essentially inconclusive. In 5.2.1 there is little effect of removal on the degree of specific lysis at any of the E/T ratios and in 5.2.2 there is a progressive reduction in specific lysis with two absorptions on monolayers but there were unfortunately insufficient cells to carry out both K-cell assays and receptor analysis so one cannot be certain that EAC3 rosettes were also reduced.

Some further limited experiments (not shown) demonstrated that specific lysis was more usually reduced following EAC3 monolayer absorption. It may be that the complement coating of the RBC is not ideal and one may tentatively conclude that the K-cell has C3 receptors.

Unfortunately insufficient cells could be recovered from the monolayers to carry out further studies on the adherent population.

Table 5.2.1 REMOVAL OF C3 RECEPTOR BEARING CELLS ON EAC3 MONOLAYERS - I

	% specific lysis at E/T of		
	10:1	5:1	1:1
unseparated (14% EAC3 rosettes)	62.4	61.7	29.9
C3 depleted* (4% EAC3 rosettes)	69.5	71.4	25.5

4×10^4 O R_1R_1 RBC per culture: adherent monocytes removed.

18 hr. cultures

* depletion as per methods section

Table 5.2.2 REMOVAL OF C3 RECEPTOR BEARING CELLS ON EAC3 MONOLAYERS - II

	% specific lysis at E/T of		
	10:1	5:1	1:1
unseparated	63.2	28.0	-4.4
C3 depleted* (1st absorption)	43.6	4.9	5.1
C3 depleted* (2nd absorption)	20.8	-	-

4×10^4 O R_1R_1 RBC per culture: adherent monocytes removed

18 hr. cultures

* depletion as per methods section

6.0 EFFECT OF DEPLETING Fc RECEPTOR-BEARING CELLS

There is circumstantial evidence in previous sections that the K-cell lysing anti-D coated RBC also has Fc-receptors and this was investigated more formally by EA-monolayer depletion experiments. The results of three experiments are shown in tables 6.0.1, 6.0.2 and 6.0.3. In table 6.0.1 it can be seen that there is virtually complete abolition of ADCC activity after passage over the EA monolayer, but some 25% reduction in % specific lysis after plain sheep RBC passage. The EA rosettes are also reduced in proportion indicating a positive correlation with specific lysis. With the donor used for the results in table 6.0.2 there was a 46.9% reduction in % specific lysis at an E/T ratio of 10:1, 38.5% reduction at 5:1 and there was only background activity at 1:1. The reduction in specific activity correlates with a reduction in EA rosettes from 15% to 5% indicating a depletion of Fc receptor bearing cells. Similar results were obtained in table 6.0.3 where there was a reduction of 24.7% in specific lysis at an E/T of 10:1, but very little difference at 5:1 and 1:1. Unfortunately there were insufficient cells in this experiment to estimate the efficiency of EA rosette depletion, which may have been poorer than in the previous experiment.

It proved impossible to recover sufficient mononuclear cells from the monolayers to carry out investigations into the putative K-cell, but preliminary microscopic observations on D-anti-D monolayers indicated the presence of

Table 6.0.1 REMOVAL OF Fc-RECEPTOR BEARING CELLS ON EA-MONOLAYERS - I

	% specific lysis
unseparated (19% EA rosettes)	35.4
Fc depleted (9% EA rosettes)	0.9
Control** (14% EA rosettes)	26.9

4×10^4 O R_1R_1 RBC per culture: adherent monocytes removed. E/T 10:1

18 hr. cultures

** passage over plain SRBC monolayer

Table 6.0.2 REMOVAL OF Fc-RECEPTOR BEARING CELLS ON EA-MONOLAYERS - II

	% specific lysis at E/T of		
	10:1	5:1	1:1
unseparated (15% EA rosettes)	75.1	33.5	7.1
Fc depleted* (5% EA rosettes)	39.9	20.6	5.3

4×10^4 O R_1R_1 RBC per culture: adherent monocytes removed.

18 hr. cultures

* as per methods section

Table 6.0.3 REMOVAL OF Fc RECEPTOR BEARING CELLS ON EA-MONOLAYERS - III

	% specific lysis at E/T of		
	10:1	5:1	1:1
unseparated*	63.2	28.0	-4.4
Fc depleted*	47.6	26.9	8.7

* insufficient cells for EA rosette estimation

4×10^4 O R_1R_1 RBC per culture: adherent monocytes removed

18 hr. cultures

mononuclear cells able to lyse parts of the monolayer - "plaque-forming" cells - which consisted of 1-10% of the added cells. Similar observations have been noted by Wahlin et al (1976) with bovine RBC, Inglis et al (1975) with sheep RBC and Biberfield et al (1975) with chicken RBC monolayers. The adherent lytic cells observed during the course of the active experiments are therefore probably the effector K-cells.

It is known that the signal for ADCC lysis requires an intact Fc part of the IgG antibody molecule (chapt. I, table 2.3.1) and the above experiments therefore demonstrate that the effector K-cell for anti-D coated RBC behaves *in vitro* in a similar manner to the K-cells identified by other systems and is likely to be the same cell.

7.0 EFFECT OF ANTI-MONOCYTE SERUM

Studies on mouse spleen effector cells have suggested that a non-phagocytic but adherent monocyte is the lytic cell with chicken RBC targets (Greenberg et al 1973). In view of the fact that human RBC are readily lysed by mature monocytes (Holm 1972) it is possible that the lymphoid K-cell described in the present experiments is a monocyte precursor, rather than a B-cell precursor.

The effects of a heterologous anti-monocyte serum (AMS) was therefore tested. After incubation in either AMS or normal rabbit control serum mononuclear cell suspensions were subjected to complement mediated lysis. It can be seen in table 7.0.1 that this was effective in reducing the number of monocytes from 14.5% to 1.0% whereas the NRS serum had little effect. It is possible that the residual monocytes after AMS were non-viable since overall viability was reduced and the suspensions were completely ineffective in lysing anti-D coated RBC compared with the NRS control cultures.

A more extended range of E/T ratios was tested in the experiments shown in table 7.0.2 and again there is complete abrogation of specific lysis after AMS but a normal dose-response curve with NRS-treated suspensions. On this occasion 6.7% recognisable monocytes were still present after AMS, but again these cells must have been inactivated even though they have not been lysed

by AMS and complement.

In table 7.0.3 the majority of monocytes were removed by prior passage over nylon wool columns, and only 1% monocytes remain. The AMS was diluted 10 fold for this experiment and post treatment viability is high. The most striking feature is the discrepancy between the usual morphological assessment of monocytes and the number of cells identified as monocytes by AMS. Unfortunately functional assays and esterase estimations were not done but from previous experience one could expect very few esterase positive cells. It is therefore possible that the effector cell possesses surface antigen specific for cells of the monocyte series and although non-phagocytic and non-adherent should nevertheless be considered as a monocyte precursor.

Table 7.0.1 EFFECT OF ANTI-MONOCYTE SERUM - I

	% monos	% viability*	% S.L.**
+ AMS treated	1.0	87	1.8
++ NRS treated	14.5	95	31.2

* ADCC assays adjusted for viability

** specific lysis at E/T ratio 10:1 (4×10^4 RBC):
Louden anti-D serum 1/3 in culture

\+ anti-monocyte serum

++ normal rabbit serum

Table 7.0.2 EFFECT OF ANTI-MONOCYTE SERUM - II

E/T	% specific lysis after	
	AMS treatment*	NRS treatment**
10:1	5.6	73.3
5:1	2.6	64.5
1:1	3.8	28 7
1:5	-0.5	9.2
1:10	-	3.7

4 x 10^4 RBC per culture : 18 hr. incubation : Louden anti-D serum 1/3 in culture.

* treated with anti-monocyte serum (6.7% monocytes)

** treated with normal serum (control) (17% monocytes)

adherent monocytes not removed prior to assay (13.5% M)

Table 7.0.3 EFFECT OF ANTI-MONOCYTE SERUM - III

	% monocytes		
	viability	phase contrast	AMS fluorescent
*AMS treated	100	0.5	3.5
*NRS treated	100	1.0	12.5

* after nylon wool passage

SECTION IV - SOME APPLICATIONS OF THE K-CELL ASSAY

SECTION IV - CONTENTS

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1.0 EFFECT OF D ANTIGEN "DOSAGE" (ZYGOSITY)

It is known that the D antigen density on the RBC surface varies depending on the rhesus genotype of the red cell and the homozygous (D/D) red cells bear more D antigen sites than the heterozygous (D/d) red cells. Even for homozygous RBC, the D antigen density varies with the full rhesus genotype and depends on the inheritance of other alleles of the rhesus system eg. R_2R_2 red cells have more D antigen sites than R_oR_o red cells (Mollison 1972, p. 270); these differences cannot normally be demonstrated by differences in agglutination titres with antibodies of appropriate specificity except in certain rare circumstances, or if special technology such as the use of a ^{125}I-anti-immunoglobulin double layer technique is used (Rochna & Hughes-Jones, 1965). It is also possible to show broad dosage differences by the use of a very sensitive autoanalyser technique (Greenwalt & Steane 1970) but the degree of individual variation is large and one individual heterozygote may have as much detectable D antigen sites as another of homozygous phenotype/ genotype.

With these limiations in mind it was decided to investigate the effect of K-cell lysis on heterozygote and homozygote D positive RBC since preliminary work had shown it to be a technique with greater sensitivity than simple agglutination methods.

The results obtained in this series of experiments under standardised culture conditions are shown in table 1.0. In two out of four cases there was a

significant difference (>10% specific lysis) between the homozygote and heterozygote RBC. In all these experiments presensitised RBC were used in the hope that the amount of anti-D absorbed by each RBC type would be proportional to the D antigen dosage. Variation in the experiments may be due to individual variation in K-cell activity as noted previously.

It seems likely that for a dosage effect to be shown with different RBC the K-cell conditions should be as sensitive as possible so that any unfavourable changes in the effector cells, antibody or target cells will produce large differences in the degree of specific lysis at the end of culture.

Therefore using presensitised RBC (which produce less lysis than with free anti-D in culture (see section I, para 7.0), the following alternatives were investigated:

1. Altering the E/T ratio
2. Shorter incubation time
3. Enhancement of cell-to-cell contact by centrifugation

Table 1.0 EFFECT OF D ANTIGEN "DOSAGE" ON K-CELL LYSIS

E/T* ratio	Lym. donor group	anti-D source	% specific lysis with O R_1R_1	% specific lysis with O R_1r
1. 10:1	AB R_1R_1	BTS 111 1/10 presens.	70.5	30.0
2. 10:1	O R_1r	IgG II presens. (neat)	63.6**	23.7
3. 10:1	O R_1R_1	IgG II presens. (neat)	81.6	77.1
4. 10:1	O rr	IgG III presens. (neat)	34.0**	33.0

* Effector:Target cell ratio. (1: 1 x 10^5 RBC; 2-4: 0.4 x 10^5RBC)

** Same RBC donor

18 hr. culture : adherent monocytes removed.

1.1 Alteration of E/T ratio

The results obtained with one individual are seen in table 1.1 (donor 4, table 1.0). There is some discrimination between homozygous and heterozygous RBC at the 5:1 ratio with 23.2% S.L. with R_1R_1 cells and 17.5% S.L. with R_1r cells (this being a 24.7% reduction with the heterozygous RBC). However at the other ratios of 10:1 and 1:1 there is no discrimination, whereas at 1:5 and 1:10 there is no detectable lysis with the R_1r cells and only minimal lysis of R_1R_1 cells.

1.2 Alteration of incubation time

The results of three experiments with short incubation times in culture are shown in tables 1.2.1, 1.2.2, 1.2.3, where different donors have been used at a single E/T ratio (10:1). A dosage effect is seen but the pattern is different for each of the individuals tested. In table 1.2.1 a dosage effect is seen both at $2\frac{1}{2}$ hours and 5 hours which is greater at the shorter incubation where 40.6% S.L. is seen with the R_1R_1 target and only 17.2% S.L. with the R_1r target. After 5 hours culture there is an increase in overall specific lysis but the difference between the homozygous and heterozygous cells is maintained (although the difference is slighly less, with 71.7% S.L. with R_1R_1 and 63.2% S.L. with the R_1r cells). With the donor in table 1.2.2, there is very little difference seen between the homozygous and heterozygous RBC at all times studied (at 18 hr: 81.6% S.L. with R_1R_1 and 77.1% S.L. with R_1r). In this particular experiment anti-D was present in culture throughout the incubation period. In table 1.2.3 two incubation

Table 1.1 EFFECT OF D-ANTIGEN ZYGOSITY ON K-CELL LYSIS-ALTERATION OF EFFECTOR/TARGET RATIO

E/T ratio	specific lysis with	
	D- HOMOZYGOUS (O R_1R_1)	D- HETEROZYGOUS (O R_1r)
10:1	34	33
5:1	23.2	17.5
1:1	10.2	12.1
1:5	6.6	-0.8
1:10	3.2	0.3

4×10^4 RBC per culture, presensitised with IgG III anti-D; adherent monocytes removed; 18 hr. culture.

Table 1.2.1 EFFECT OF D-ANTIGEN ZYGOSITY ON K-CELL LYSIS-ALTERATION OF INCUBATION TIME - I

INCUBATION TIME (HRS)	specific lysis with	
	D HOMOZYGOUS (O R_1R_1)	D HETEROZYGOUS (O R_1r)
0	0.6	-4.9
2.5	40.6	17.2
5.0	71.7	63.2

E/T 10:1 (4×10^4 RBC per culture) : presensitised with IgG III anti-D, adherent monocytes removed.

Table 1.2.2 EFFECT OF D-ANTIGEN ZYGOSITY ON K-CELL LYSIS-ALTERATION OF INCUBATION TIME - II

INCUBATION TIME (HRS)	specific lysis with	
	D- HOMOZYGOUS (O R_1R_1)	D- HETEROZYGOUS (O R_1r)
0	0.6	-0.8
2	64.6	59.0
6	73.3	72.5
18	81.6	77.1

E/T 10:1 (4 x 10^4 RBC per culture) ; IgG III anti-D 1/3 free in culture, adherent monocytes removed.

Table 1.2.3 EFFECT OF D-ANTIGEN ZYGOSITY ON K-CELL LYSIS-ALTERATION OF INCUBATION TIME - III

INCUBATION TIME (HRS)	specific lysis with	
	D-HOMOZYGOUS (O R_1R_1)	D-HETEROZYGOUS (O R_1r)
0	2.8	0
4	10.2	-0.3
18	63.6	23.7

E/T 10:1 (4 x 10^4 RBC per culture) presensitised with IgG III anti-D adherent monocytes removed.

times were studied and with this particular donor a clear dosage-effect showed with a 63.6% S.L. with R_1R_1 cells and 23.7% S.L. with R_1r cells after 18 hr. culture, but at 4 hours the R_1r cell has not been lysed above background and only 10.2% S.L. was seen with the R_1R_1 cell.

Examining cultures at incubation times less than 18 hours does in some cases allow discrimination between homozygous and heterozygous D positive red cells especially if presensitised with anti-D, but again the individual variation is such that zygosity could not be determined with any degree of certainty in a case where the genotype is unknown.

1.3 Centrifugation contact

In an attempt to increase the effect of detection of zygosity, a combination of short incubation with centrifugation-enhanced contact (see section II, para 2.0) was employed, as opposed to the standard culture conditions where cells were allowed to settle under gravity. The results are shown in fig. 1.3. It can be seen that at both incubation times of 2.5 and 5 hours, centrifugation at the beginning of the culture period significantly enhanced the specific lysis seen with both the R_1R_1 and R_1r targets, and that a clear-cut dosage effect is seen at both incubation times. The lysis of R_1r cells allowed to settle under gravity in this particular instance is very poor (compared with results in table 1.2.3 at the comparable incubation time of 4 hours). This particular RBC donor may have had particularly low numbers of D antigen sites which

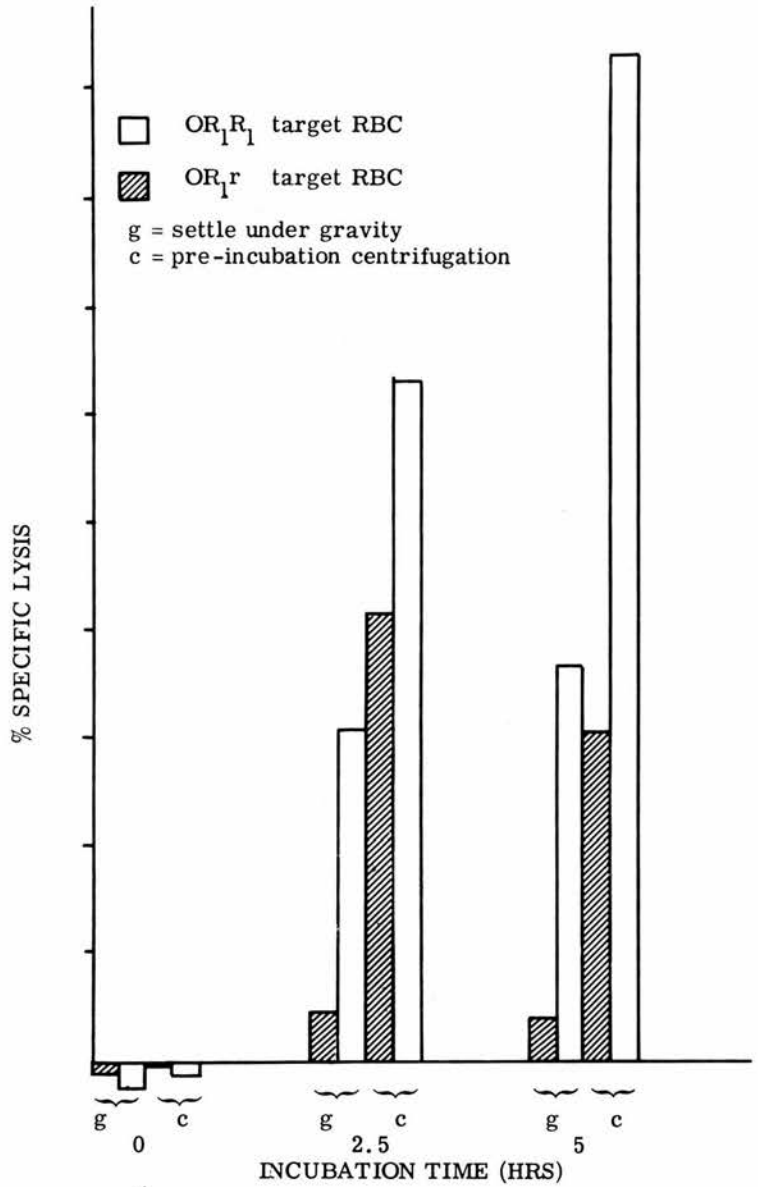

OR1R1 target RBC
OR1r target RBC
g = settle under gravity
c = pre-incubation centrifugation
% SPECIFIC LYSIS
g
c
0
g
c
2.5
g
c
5
INCUBATION TIME (HRS)
4x10^4 RBC per culture. E/T 10:1
RBC presensitised with IgGIII anti-D
adherent monocytes removed

Fig. 1.3

allowed good discrimination of K-cell lysis.

1.4 Conclusions

As a result of various manipulations of the K-cell culture system, dosage effects between heterozygous and homozygous D positive cells could be shown. This further re-inforces the evidence that the specificity for K-cell lysis resides with the appropriate antigen/antibody combination and not with the K-cells themselves. The individual variations seen with different red cell donors when using these modifications of the K cell system means that this particular assay cannot be used to detect zygosity with any degree of certainty.

2.0 CYTOLYTIC ACTIVITY OF ANTI-D FROM DIFFERENT SOURCES

Since the majority of preliminary experiments involved in demonstrating the existance of K-cell lysis used a single donor of anti-D, it was important to demonstrate that anti-D from other sources were equally capable of specific lysis of D positive RBC. Six random anti-D samples used for routine work in the Blood Transfusion laboratories were assessed for cytolytic activity and the results are shown in table 2.0.1. It can be seen that specific lysis of D positive RBC occurs with all samples tested but it is interesting to note that the degree of lysis bears no relationship to the anti-D titres. It is relevant that the anti-D materials are in fact each prepared from a random pool of donors thus any individuals will be masked.

The cytolytic activity of a variety of anti-D sera obtained from single individuals were therefore assessed and the results are expressed in fig. 2.0 and tables 2.0.2 and 2.0.3. On these occasions, the concentration of anti-D has been estimated in μg/ml. The concentrations of anti-D were equalised so that, nominally, there was an equivalent amount of anti-D per culture from each of the different individuals.

In fig. 2.0 comparable specific lysis is obtained with anti-D from each of the three individuals, and with dilution, the specific lysis is reduced a similar amount.

Similar results are obtained in table 2.0.3 where, again, there is widely differing cytolytic activity

at comparable anti-D concentrations. The results in Table 2.0.2 were a little more confusing in that the degree of specific lysis does not correlate well at different anti-D dilutions since there is an apparent increase in K-cell lysis at 0.6 µg/ml. This may be related to an undetermined artefact resulting from dilution of the original anti-D to achieve the given concentration, since there is an apparent inverse correlation between the original anti-D concentrations and the % S.L. achieved at 0.6 µg/ml in culture, and in fig. 2.0 where the original starting anti-D concentrations are very similar, approximately parallel lines with dilution are obtained with each serum.

Despite these discrepancies, it is apparent that anti-D from different individuals behaves differently in the K-cell assay. The biological difference does not appear to be related to whether or not the anti-D is produced "naturally" by immunisation during pregnancy or "artificially" by the deliberate injection of Rhesus negative male volunteers, since two out of three male anti-D's showed biological low activity compared with anti-D quantitation and two out of five female anti-D's gave low results compared with quantitation.

It may be that the IgG sub-class distribution of these anti-D's vary and that this is responsible for the different lytic activity of a given agglutinating quantity of anti-D. This has not been verified experimentally however.

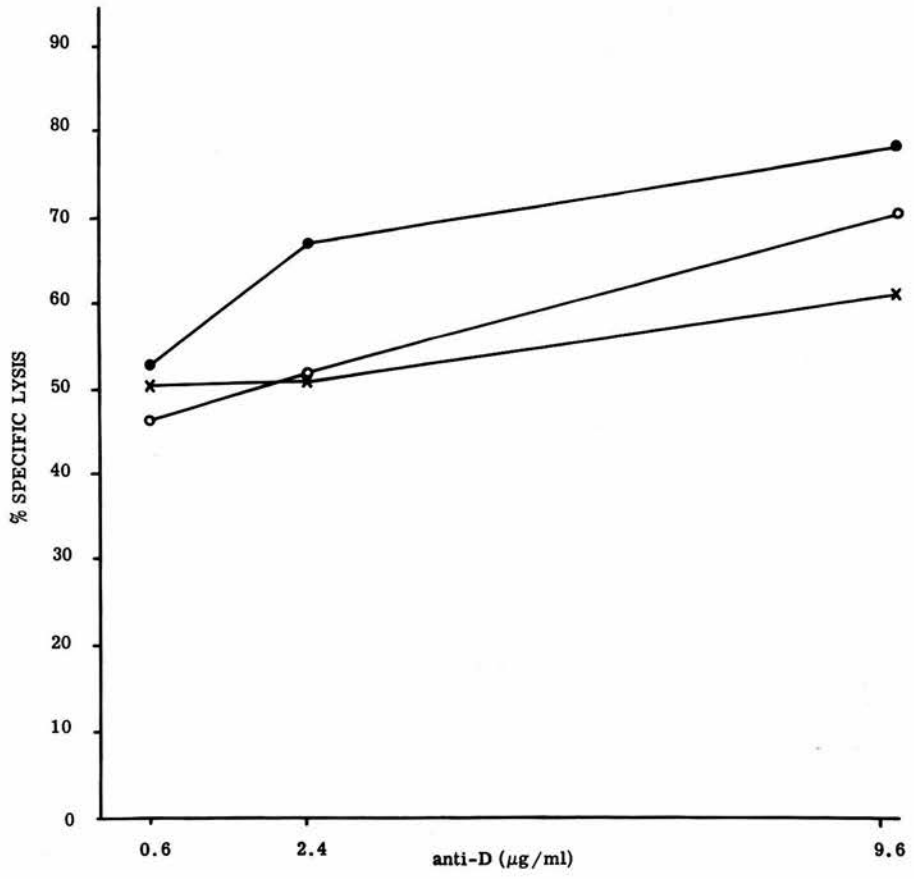
Table 2.0.1 CYTOLYTIC ACTIVITY OF ANTI-D FROM DIFFERENT SOURCES

anti-D source	% S.L.*	anti-D titre**
BTS 155	66.5	1/64
BTS 167	53.5	1/32
BTS 184	41.7	1/64
BTS 207	33.3	1/64
BTS 210	46.3	1/32
BTS 221	50.8	1/128
AB serum	4.6	nil

* %specific lysis with anti-D added to cultures - diluted x 6.

** papain titre vs. O R_1R_1 RBC
E/T ratio 10:1 (1×10^4 O R_1R_1 RBC per culture)
18 hr. culture; adherent monocytes removed.

CYTOLYTIC ACTIVITY OF DIFFERENT ANTI-D SERA ADJUSTED TO EQUIVALENT ANTI-D CONCENTRATION



● I.P. ♀ anti-D : original conc^{n} 33.7 μg/ml

o L.K. ♀ anti-D : " " 30 μg/ml

x W.McP anti-D : " " 31.1 μg/ml

E/T ratio 10:1 ($4x10^{4}OR_{1}R_{1}$ RBC per culture) : adherent monocytes removed; 18hr. culture with final anti-D conc^{n} as shown.

Fig. 2.0

Table 2.0.2 CYTOLYTIC ACTIVITY OF DIFFERENT ANTI-D SERA

ADJUSTED TO EQUIVALENT ANTI-D CONCENTRATION - II

Source of anti-D	papain titre	IAGT titre	original serum anti-D concentration (μg/ml)	μg/ml anti-D per culture			
				12.0	2.4	1.8	0.6
D.H. ♂	1/256	1/1024	38.9	58.4*	58.7	57.8	64.4
O.H. ♂	1/1024	1/63,536	809.5	62.8	-	-	16.6
I. McC. ♀	1/64	1/128	6.6	-	-	75.3	85.9
H.L. ♀	1/32	1/256	4.7	-	-	73.8	85.5
S.L. ♀	1/64	1/128	8.11	-	92.0	72.1	82.4

* % specific lysis under conditions below
E/T 10:1 (4×10^4 RBC per culture) adherent monocytes removed;
18 hr. cultures with anti-D concentrations as shown
- = not tested

TABLE 2.0.3 CYTOLYTIC ACTIVITY OF DIFFERENT ANTI-D SERA

ADJUSTED TO EQUIVALENT ANTI-D CONCENTRATION - III

source of anti-D	anti-D concentration in original serum			μg anti-D per culture		
	papain titre	IAGT titre	analyser μg/ml	7.0	3.5	1.0
D.F. ♂	1/256	1/1024	23.2	82.8	76.5	82.4
L.K. ♀	1/256	1/256	30.0	23.2	35.7	18.0
L.C. ♀	1/8	1/32	4.4	-	-	41.2

* % specific lysis under conditions below
E/T 10:1 (4×10^4 RBC per culture) adherent monocytes removed
18 hr. cultures with anti-D concentrations as shown.

3.0 INDIVIDUAL DONOR VARIATION IN K-CELL ACTIVITY

During the course of the preliminary experiments defining the K-cell assays it was noted that certain individuals gave rather "poor" responses, and others, consistently "good" responses in terms of specific lysis obtained under standard conditions.

It has been noted that females have lower K-cell activity than males when human lymphocytes target cells are used (Kovithavongs et al 1974) but that there is no sex difference in lytic potential with human RBC targets (Kovithavongs et al 1975).

In fig. 3.0 the results obtained with 4 individuals over a two year period are shown. The assay systems are comparable in that the E/T was 10:1 and an excess of Louden anti-D was used as antibody. It can be seen that two of the individuals (donors 1 and 2) there is a tendancy to consistently high activity with certain fluctuations; these might be termed "good" K-cell donors with specific lysis in the region of 60-80% S.L. Similar results were obtained with several other individuals (insufficient data for full plot) suggesting that this is a general phenomenon.

Conversely donor 3, in general, shows rather poor cytolytic potential, never reaching values above 50% specific lysis. Again, this was seen with other individuals who of course were not bled very often because of the poor responses. It is probably co-incidence that donor 3 is a female since similar "poor" activity has been observed with male donors.

Donor 4 appears intermediate in that occasional very high and low results are obtained.

It is interesting to note the occasional sudden increase or decrease in activity in all of the individuals tested over a long period of time. As far as can be ascertained these changes do not coincide with any changes in methodology and in any case, rising activity is seen with some donors at the time as a fall in activity of another (see fig. 3.0). This suggests that the changes observed are "real" but the significance of these fluctuations is undetermined. It may be that minor infections or changes in health affect K-cell activity similar to the fluctuations observed in lymphocyte transformation in individuals over a period of time (Dr. A.G. White & R. Barclay unpublished observations).

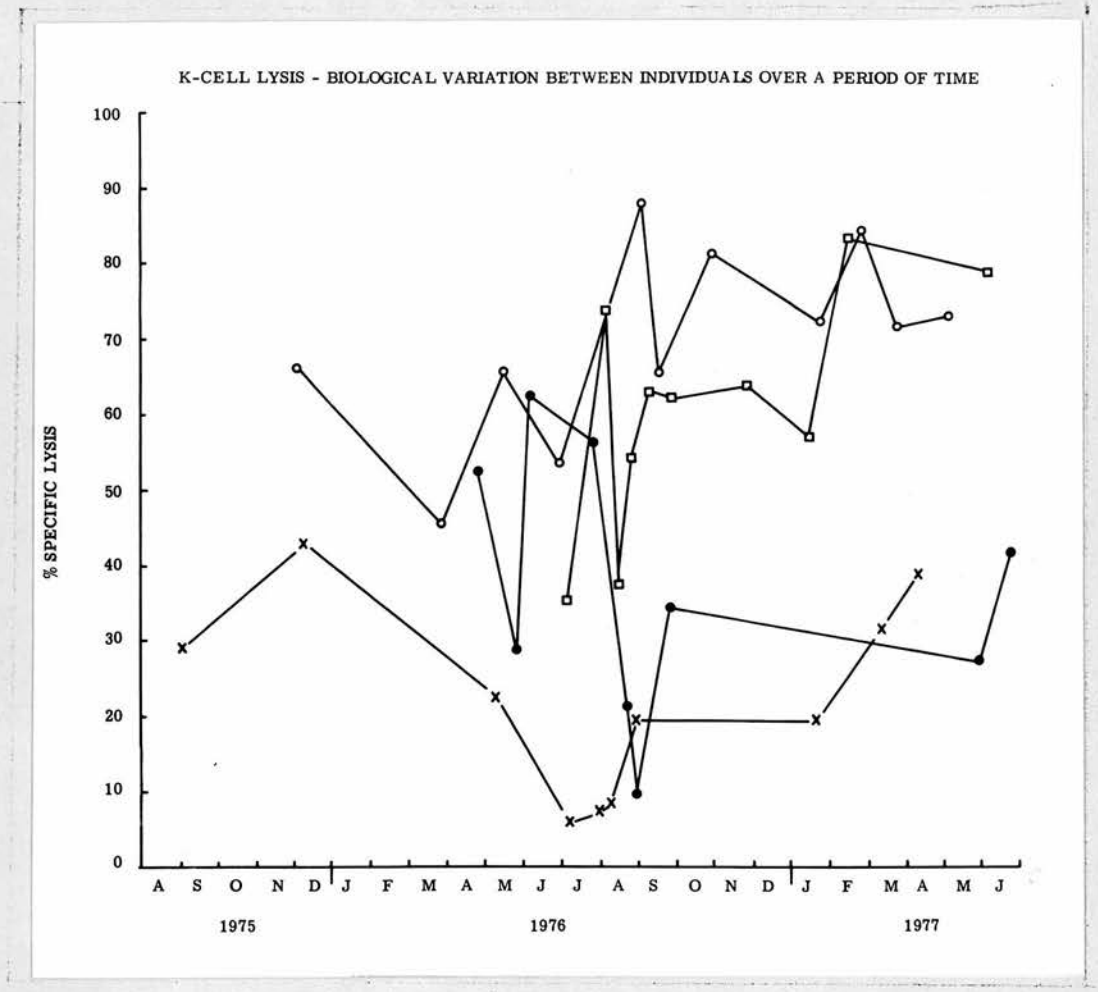


Fig. 3.0

□ – □	donor 1	M.M.	♂
○ – ○	donor 2	P.T.	♂
× – ×	donor 3	GL.	♂
● – ●	donor 4	M.G.	♀

4.0 K-CELL ACTIVITY OF CORD BLOOD MONONUCLEAR CELLS

It has been shown that the cord blood of neonates contains a population of K-cells able to lyse sensitised Chang cells (Campbell et al 1974) indicating the presence of K-cells at birth.

If it is postulated that foetal K-cells play a role in the lysis of anti-D sensitised RBC in haemolytic disease of the newborn, then it is necessary to demonstrate the ability of neonatal mononuclear cells to lyse these cells.

The results of *in vitro* tests on the cord blood mononuclear cells from 5 neonates is shown in table 4.0. Suspensions have been tested with and without removal of adherent monocytes over a range of E/T ratios.

In some respects the results are quite different from the adult pattern. In all donors except no. 1 there is very little activity in the suspensions where monocytes have *not* been removed, even at the highest E/T tested (10:1) where the maximum % specific lysis observed is 28.8% S.L. In contrast, the monocyte-depleted populations all show significant activity, almost comparable to the adult levels (see section I, fig. 5.2.2) and there is a similar fall in % specific activity as the E/T is reduced. It can be calculated from the dose response curve (not shown) that 50% specific lysis is seen at approximately 50 x 10^4 mononuclear cells per culture whereas with adult cells over the same E/T ratios and same source of anti-D (Louden serum) the 50% lysis point is obtained

with 22 x 10^4 mononuclear cells per culture (figure not shown). It would appear therefore that the monocyte-depleted neonatal cultures have approximately half the adult lytic capacity when tested under the same condition.

The reason for the poor activity with monocyte-rich cultures is a matter of conjecture - it may be that the cord blood K-cells are greatly concentrated by removal of monocytes since the % monocytes were rather higher than seen with adults (mean of 21% compared to 15%). In the one case where significant pre-nylon activity is seen (donor 1) the monocytes were reduced only from 7.1% to 2.7% whereas with donor 4 who gave the highest post nylon % specific lysis of 59.9% the corresponding reduction was from 32.3% to 1.6%. It is possible to argue that the putative K-cell population has been enriched to a higher degree with donor 4 than donor 1, and similar ly for the other neonates.

It is also noted that the efficiency of removal of monocytes is not as efficient as with adults, and it may be that the neonatal cells physical characteristics (adherence) are immature, as well as the functional characteristics (lysis of RBC).

This series of experiments has demonstrated that neonates have cells with K-cell activity although the nature of these cells has not been defined as for the adults. Nevertheless, it is likely that a similar K-cell is operative as in the adults and that these cells may play a part in the lysis of sensitised RBC in haemolytic disease of the newborn.

Table 4.0 K-CELL ACTIVITY OF CORD-BLOOD MONONUCLEAR CELLS

cord blood donor	% monocytes		% specific lysis at E/T of							
	pre	post	10:1		5:1		1:1		1:10	
1. B.McP. ♀	7.1	2.7	28.8	42.8	21.8	16.0	1.9	4.1	-1.3	2.4
2. B.McK. ♂	20	3.6	-0.5	33.3	-1.2	9.8	3.9	-0.1	-4.3	-2.7
3. B.M. ♂	14.3	3.5	-1.1	40.8	4.2	38.6	-1.3	5.6	-3.2	-0.4
4. B.D. ♀	32.2	1.6	-6.5	59.9	4.3	40.5	0.3	9.7	-3.4	-1.3
5. B.S. ♀	26.7	4.4	1.3	56.0	-0.6	40.9	-1.9	6.6	-1.6	-1.1

4×10^4 O R_1R_1 RBC per culture

Louden anti-D serum 1/3 free

18 hr. cultures

5.0 EFFECTS OF ADRENALIN INFUSION ON K-CELL ACTIVITY

It has been shown that the parenteral infusion of adrenalin into healthy normal subjects produces a leucocytosis which occurs in two stages 1) an early phase with a rise in circulating lymphocytes at 15 - 30 mins and 2) a late phase at 1 - 3 hours where the lymphocyte count returns to normal but a neutrophil leucocytosis is seen (Steel et al 1971; Gader 1974; Gader & Cash 1975). Similar changes have been noted following exercise (Steel et al 1974), and the lymphocytosis is mainly due to a relative increase in B lymphocytes (Steel et al 1974; Hedfors et al 1975). Hedfors et al (1975) also showed that there was an increase in B lymphocytes and in Fc bearing lymphocytes. Since exercise is associated with an increase in the blood level of adrenalin (Kotchen et al 1971) it is possible that the above changes are reflections of a common mechanism. It has been shown in animals that adrenalin infusion increases the thoracic duct lymph output (Doemling & Steggarda 1962) and the release of lymphocytes from the spleen (Ernström & Söder 1975) and it is therefore likely that above noted changes in peripheral blood leucocytes occur as a result of mobilisation and redistribution of cells.

Adrenalin infusions were undertaken jointly with Dr. Gader and R. Barclay (in our department) as a continuation of the above studies, and the opportunity was taken to study changes in K-cell function as well as T and B lymphocyte numbers.

Adrenalin was infused intravenously at a rate of 7 μg/min for 30 min as reported previously (Gader & Cash 1975) and the results of one infusion are shown in table 5.0 and fig. 5.0.

In table 5.0 it can be seen that there is a marked leucocytosis at 15 and 30 min which is almost entirely due to an increase in the numbers of lymphocytes, and that the values have returned to normal limits by 60 mins (ie. 30 mins following infusion). The lymphocytosis is due to an increase in the absolute numbers of both T cells and of B cells. The relative increase in T cells is only $2\frac{1}{2}$ times higher than the resting value, whereas the B cell increase is some $5\frac{1}{2}$ times higher at the peak response. Similar findings were reported by Steel et al (1974) who observed an 8-fold increase in non-T cells but a $2\frac{1}{2}$ times increase in T cells at 15 mins after the commencement of vigorous exercise, and by Hedfors et al (1975) who showed a two-fold increase in T cells and $2\frac{1}{2}$ times increase in B cells.

The K-cell results are shown in fig. 5.0 where ADCC activity has been estimated at the same times as the leucocyte counts, and over a range of E/T ratios from 1/10 to 10/1. This particular subject has very high % specific lysis, in the region of 90% S.L. at 10/1, and it can be seen that there is an increase in % specific lysis during the course of the infusion (maximum at 15 min) which is seen best at the lower E/T ratios where there are less K-cells present initially. The change in specific lysis parallels the observed changes in B

cells as noted by Hedfors et al (1975). It should be noted that it is the relative percentages of T and B cells which are important in this functional assay since the cell suspensions obtained at each point in time are adjusted to give the same numbers of mononuclear cells in culture. It is therefore relevant that an increase in % B cells was noted above, and by Hedfors et al (1975). However, the B cells were identified by EAC3 rosettes and it has been shown that K-cells also possess the C3 receptor; it is therefore invalid to conclude that the K-cell in the above system is a B cell. It may be pertinent to note that % T & B always approximates to 100% when estimating B cells by EAC3 rosettes, but less than 100% when estimated by SIg receptors. This suggests that a non-T, non-B cell is being detected by EAC3 rosettes - possibly the K-cell.

The observed changes in ADCC activity are likely to be due to an increase in the numbers of such cells

rather than an increase in functional capacity; Hedfors et al 1975 reached a similar conclusion.

Table 5.0 ALTERATIONS IN LEUCOCYTE POPULATIONS DURING ADRENALIN INFUSION

	Time (mins)*			
	0	15	30	60
TWBC	3772	7538	7685	3585
Polys	1018	1688	1713	1429
Lymphocytes (total)	1682	4342	4495	1390
T-cells**	1430 (85)***	3170 (73)	3686 (82)	1251 (90)
B-cells**	236 (14)	1346 (31)	944 (21)	181 (13)

TWBC = total leucocyte count (per cu.mm)
polys = neutrophil polymorph count (per cu.mm)

* time of infusion 30 mins; 0 = pre- infusion
** estimated as in methods chapt.
*** percentage T or B cells in total lymphocyte count

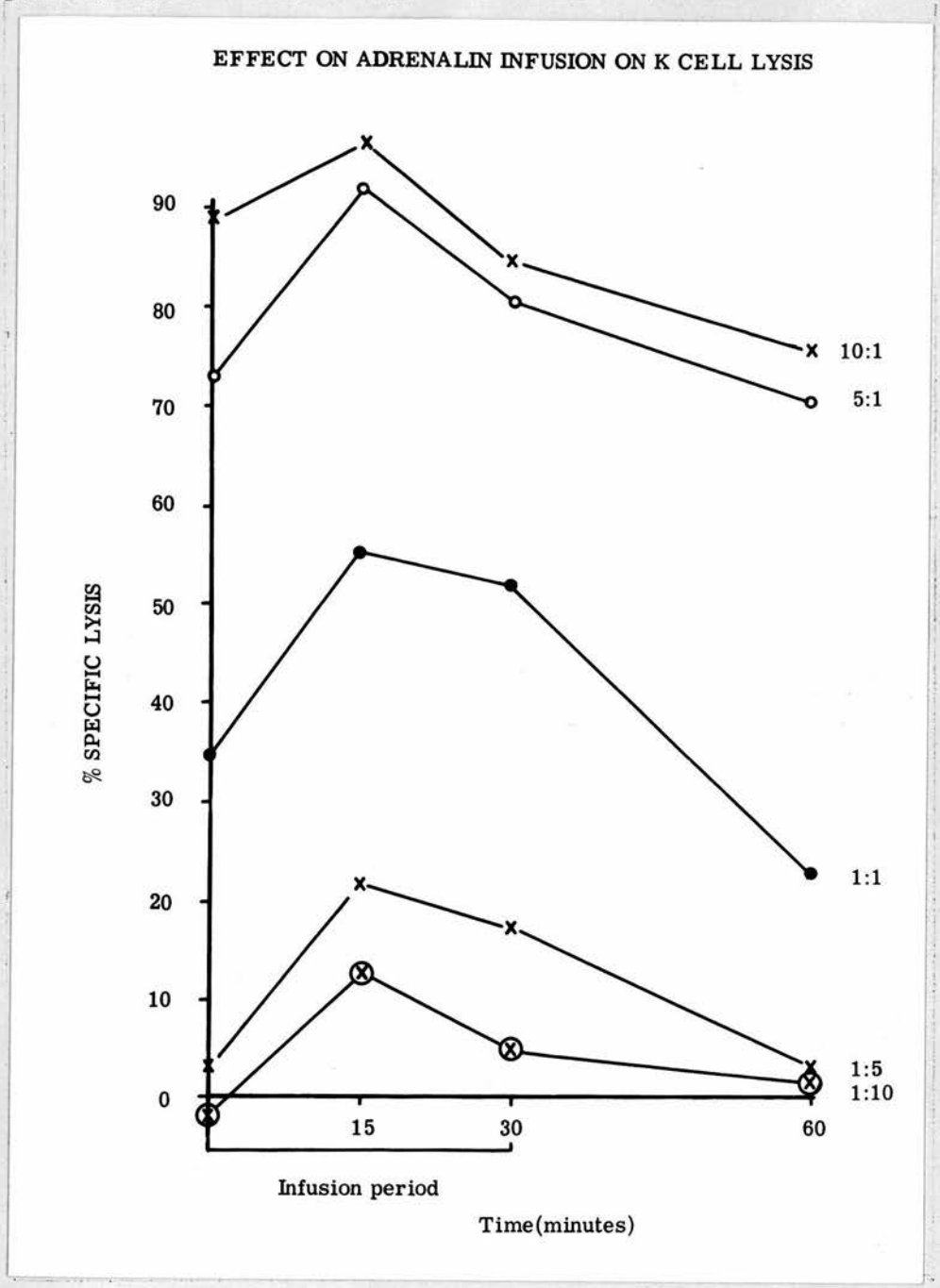

EFFECT ON ADRENALIN INFUSION ON K CELL LYSIS
% SPECIFIC LYSIS
90
80
70
60
50
40
30
20
10
0
10:1
5:1
1:1
1:5
1:10
15
30
60
Infusion period
Time(minutes)

Fig. 5.0

6.0 EFFECT OF INFUSION OF D-POSITIVE RBC INTO D-NEGATIVE MALE VOLUNTEERS

At the Regional Transfusion Centre, Rhesus (D) negative male volunteers are regularly injected with D-positive RBC for the production of anti-D for therapeutic use. It is known that approximately 70% of persons so injected will respond satisfactorily to produce useful levels of antibody (Gunson et al 1976). At present however there is no way of identifying "responders" from "non-responders" as regards the production of suitable levels of anti-D apart from a series of time-consuming injections. Several volunteers were therefore studied over a period during which they received a single unit of frozen-thawed blood (200 ml D positive RBC), rested for 6 months and then received booster injections of 1.0 ml D-positive RBC once per month.

To date, three volunteers have been studied from the time of their 1st injection and all have shown a similar pattern of K-cell activity as assessed by the "standard" technique of E/T ratio 10:1 with an excess of Louden anti-D serum as antibody source. The results obtained with one of these donors is seen in fig. 6.0 There is a transient fall in K-cell activity during the primary stimulus which is seen at 12-14 days (at 12 days in volunteer 2). During this time the 50% lysis point has decreased from approximately 1.3×10^4 effectors/ culture to 4.3×10^4 effectors per culture. The K-cell activity then returns to greater than normal seven weeks after the infusion so that only 0.1×10^4

effectors/culture produce 50% specific lysis. It is interesting to note that the plateau in % specific lysis is the same as pre-infusion suggesting that the total capacity for lysis is the same. It is therefore possible that the increase in the 50% lysis point at 33 days is due to increased functional capacity rather than an increase in the numbers of K-cells.

The transient fall in K-cell activity at 12 days is of interest since it corresponds with the time of primary immunisation. It is unlikely however that K-cells are directly involved and that this is a secondary phenomenon. The reduction in % specific lysis is considerable and the plateau of activity seen at other times is not approached. This would suggest that there is a reduction in K-cell numbers as well as function since the total lytic capacity is reduced. It is possible that there has been redistribution of K-cells eg. to the spleen, where there will be increased requirements for the removal of the infused D-positive RBC.

It would be of importance to see whether this pattern of response occurs with all volunteers or whether it corresponds to responders only. At the time of writing, it is too early to assess the final antibody levels produced but it may be significant that all three volunteers studied already have detectable anti-D some 6 months after the 1st injection of D-positive RBC.

CHANGE IN K-CELL ACTIVITY FOLLOWING INFUSION OF D POSITIVE RBC INTO D NEGATIVE MALE VOLUNTEER

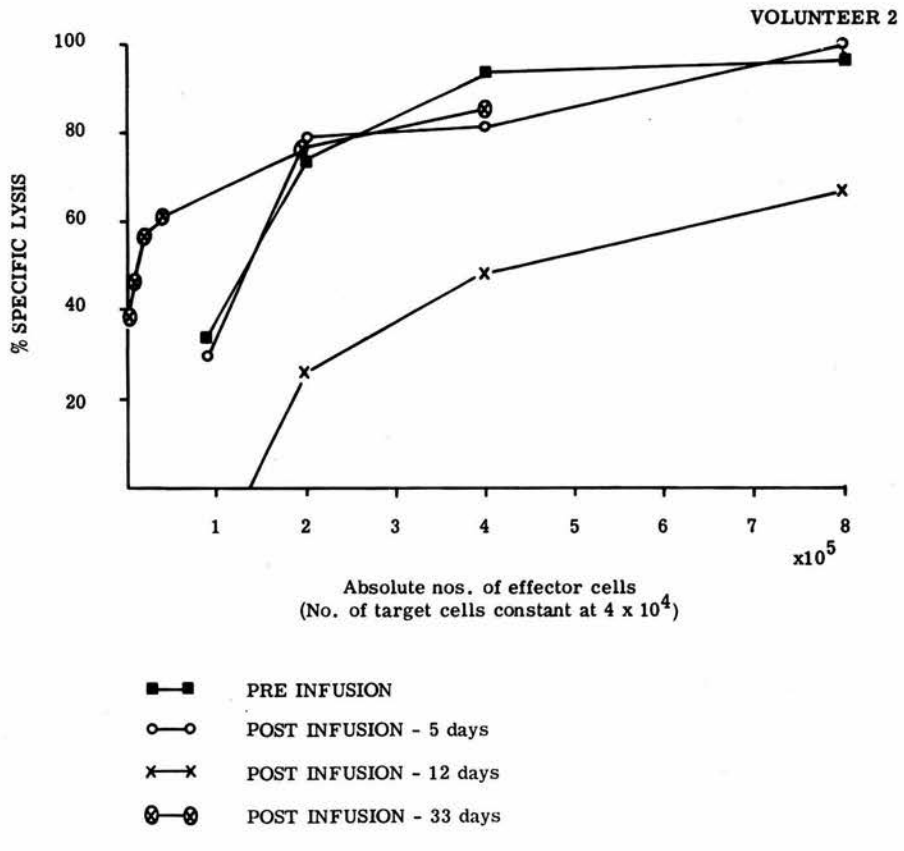


Fig. 6.0

CHAPTER V - DISCUSSION AND CONCLUSIONS

CHAPTER V - CONTENTS

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1.0 INTRODUCTION

The original aim of developing an ADCC assay using a homologous human system based on the non-monocytic K-cell has been realised. The early experiments were designed to assess the lytic potential of mononuclear cell suspensions with monocytes removed by adherence. The D-anti-D system was chosen for the reasons outlined in chapt. II and in particular, studies were done using the same source of anti-D - a woman immunised during pregnancy. This was done deliberately so that there was a minimum change in variables whilst investigating the mechanism of lysis. For the same reason group O R_1R_1 RBC were used almost exclusively as the target cell. However, the general validity of the ADCC system was later confirmed with several different anti-D's and RBC of other rhesus genotype.

2.0 INVESTIGATION OF CULTURE VARIABLES

It was shown in early experiments, in common with published evidence (Hinz & Chickosky 1972) that mononuclear cell suspensions depleted of adherent monocytes were effective in lysing anti-D sensitised RBC. The system appeared to be somewhat insensitive in that high anti-D concentrations were required, and that an effector/target ratio of at least 10:1 was usually required (table 1.0.2, p. 100). The use of proteolytic enzymes to increase ADCC activity had been reported by Holm (1972). Since papain is widely used in blood transfusion practice to enhance serological reactions (especially with antibodies of the Rhesus system) it seemed appropriate to use this enzyme. Successful enhancement of specific lysis of anti-D coated RBC was obtained (see para. 1.0, p. 95) the ^{51}Cr labelled

RBC remained stable over the period of culture (up to 18 hrs) although serum supplementation of culture medium was required. The nature of the enhancing effect is uncertain but is probably due to a combination of increased antibody uptake by the RBC (Masouredis 1962) and increased exposure of the Rhesus antigens in the cell membrane (Romano et al 1975) allowing greater presentation of activated Fc determinants to the effector K-cell. It has been reported that papainisation of monocytes enhances the binding of sensitised RBC (Kenna et al 1975) but this mechanism was not effective in the above K-cell system since papainisation reduced specific lysis (table 2.0, p. 105).

In view of the intention to use the K-cell assay for clinical investigation, it was felt important to standardise the variables as much as possible. The coefficients of variation for identical samples processed in parallel were under 10% and this was felt to be acceptable for a biological assay involving several manipulations and processings.

During the development of the K-cell assay certain methodological changes were introduced to reduce the requirements for cells and to reduce the technical manipulations required. Hence early experiments were performed in plastic test tubes and later experiments in microplates. Extensive experiments into the effect of altering culture volume ratios of effector/target cells and cell densities (para. 4.0, p. 110) revealed that the important factors were 1) the quantity of antibody present in relation to the number of RBC and 2) the relative number of effector and target cells ie. the E/T ratio. Provided that an excess of anti-D was present, the actual culture volume was not critical. This was an important practical point for the evaluation of

various inhibitors added directly to cultures. More detailed investigations into antibody requirements and effector/target ratios were reported in results section I.

Provided that there is an excess of sensitised antibody present, then the limiting factor in determining the amount of RBC lysis is the number of available effector cells. The number of effector cells is likely to remain constant during the relatively short culture incubation period of 18 hrs. and therefore when the numbers of mononuclear cells are changed relative to a given number of RBC the % specific lysis obtained is a reflection of the number of effector cells present. This is well demonstrated in fig. 5.1, p. 120 and fig. 5.2.1, p. 125 where sigmoidal dose response curves are seen with a plateau of maximum lysis being observed under the conditions used. The number of effector K-cells per culture is unknown since one is merely measuring a functional end point ie. RBC lysis. It is therefore difficult to standardise results between individuals since it is not possible to say whether variations in lysis observed due to an increased number of effector cells, or merely an increase in efficiency of a small number of cells. It is possible to partially solve this problem by calculating from the dose response curve the number of mononuclear cells per culture required to give 50% specific lysis. This approach has been used by other workers in an attempt to evaluate changes in K-cell activity (Campbell et al 1974). An alternative approach is the visualisation of the number of effector cells by using RBC monolayers and estimating the number of plaque-forming cells ie. those which have lysed surrounding RBC (Wahlin et al 1976; Biberfield et al 1975).

Current techniques are somewhat imperfect however, and errors may easily be introduced due to the difficulties involved. Furthermore, non-human RBC and sensitising antibodies have been used so that the relevance to human K-cell evaluation may be questionable.

When the effector/target ratio is altered at differing anti-D concentrations, then a family of curves is obtained (fig. 5.3, p. 123) which reflects the relative availability of antibody as well as the intrinsic activity of the effector cell populations. The antibody requirements were explored further in section I, para. 6.0, p. 129. When anti-D containing material was serially diluted in the presence of a fixed number of effector and target cells, a dose-response curve was obtained with an initial plateau of maximal lysis where anti-D was in excess, followed by a gradual decrease in % specific lysis as the antibody became rate-limiting. At the highest concentrations of anti-D there was a suggestion of a prozone phenomenon. This could be due to saturation of both the RBC target antigens and the effector cell Fc receptors by an excess of anti-D molecules. A similar antibody response has been reported by Ziejlemaker et al (1975) with a K-cell assay involving a transformed cell line.

Significant lysis of RBC was seen down to anti-D concentrations of 0.02 µg/ml in culture which corresponds to approximately 3 ng anti-D per culture. Detection of activity at this degree of dilution makes this biological assay of anti-D very much more sensitive than the conventional serological methods. It is possible therefore that in

selected instances eg. suspected haemolytic disease of the newborn, the K-cell assay would prove to be more sensitive and indeed more relevant, since the biological activity of the antibody is being assessed. This is discussed below in more detail. The effect of anti-D availability in culture is also demonstrated by experiments comparing the free anti-D with RBC presensitised by the same anti-D before being added to culture (table 7.0, p. 141). With anti-D presensitisation, the majority of D antigen sites are likely to be occupied by high affinity anti-D and efficient lysis should follow after contact with effector cells. Some anti-D will be inevitably lost during the washing processes, and further dissociation might occur during the culture time. A higher concentration of anti-D will be achieved throughout the conditions of culture where anti-D is added directly to the cultures and the relative proportions of anti-D to RBC is very much greater (0.4 - 0.1 x 10^5 RBC compared with 200 x 10^5 RBC). Nevertheless, high activities are seen with presensitisation in certain instances and this may be a reflection of the different affinities of the anti-D sera. Quantitative assessment of cell-bound anti-D by ^{125}I-antiglobulin reagent would be valuable in determining whether or not this is the case and this is a potential area for future research. In practice, the K-cell assay characteristics were similar whether or not anti-D was added directly, or used to presensitise RBC. Although a lower final % specific lysis was usually obtained, the greater sensitivity to changes of effector cell function was valuable when investigating the effects of various inhibitors (q.v.).

3.0 SPECIFICITY OF K-CELL LYSIS

During the course of many of the earlier experiments it was shown that donors of various blood groups were able to provide active K-cells against group O anti-D sensitised RBC, eg. table 4.2, p. 114, table 5.1, p. 119, table 5.2, p. 122, and table 7.0, p. 141. Indeed, in all cases tested, mononuclear cells were able to lyse autologous target cells in the presence of anti-D (eg. donor 1, table 3.1, p. 10 and donor 3, table 5.1, p. 119). Experiments designed to show acquired specificity for lysis by effector cells gave negative results (para. 6.0, p. 179). The anti-D antibody was not cytophilic and effector cells pre-incubated in anti-D and then transfered directly to target cells failed to induce lysis.

The specificity for inducing lysis does not appear to lie with the effector cell but with the appropriate combination of antibody and antigen. In several experiments reported in section I, para. 8.0 it was clearly shown that with anti-D antibody, lysis was induced only in the presence of D-positive RBC and not D-negative RBC. Furthermore the Rhesus antigens other than D did not influence specific lysis to anti-D so that efficient lysis was seen with O R_1R_1, O R_1R_2 or O R_2R_2 cell types. There was a suggestion that the D zygosity of RBC could be determined from the endpoint of lysis. This is not unexpected since the D-antigen density on the RBC will differ and hence the amount of cell-bound anti-D will vary. This was investigated further in chapter IV, p. 255.

Using the more sensitive assay with anti-D pre-sensitised RBC, consistent results were not obtained even after manipulations such as altering the E/T ratio, shortened

incubation time and enhancement of cell-to-cell contact by centrifugation. Although clear-cut D-antigen dosage effects could be obtained in individual instances, the degree of variation seen with different red cell and K-cell donors meant that the ADCC assay could not be used to detect D-antigen zygosity with any degree of certainty. It is possible however that the culture incubations could be manipulated in such a way as to make this a more reliable technique for determining D antigen zygosity and this is an area for future potential investigations. Overall, the results tend to reinforce the evidence that the specificity for K-cell lysis resides with the appropriate antigen/antibody combination and not with the K-cells.

Absorption experiments showed that only D-positive RBC removed the cytolytic activity from anti-D containing sera confirming that the appropriate combination of antibody and red cell antigen is required for ADCC activity. However, suitable rhesus antibodies other than anti-D were not obtained for studies. Appropriate positive controls to demonstrate that rhesus negative (O rr) cells can be lysed by K-cells would require cytolytic anti-c or anti-e sera. However, there is no reason to suspect any intrinsic difference in susceptibility to lysis between Rh positive and Rh negative RBC and the absence of these controls is not critical to the interpretation of the results.

4.0 MECHANISM OF LYSIS

On the basis of the previous experiments, it has been shown that antibody-coated RBC are necessary for the induction of lysis, and that the degree of lysis was influenced

by the D-antigen dosage in the RBC, and the amount of antibody present. Previously reported ADCC systems have shown that direct cell-to-cell contact is required for lysis (Perlmann & Perlmann 1970, Biberfield et al 1970). A similar mechanism appears to operate with the D-anti-D system because centrifugation prior to culture enhances the initial rate of specific RBC lysis and also increases the total % specific lysis achieved (section II, para. 2.0, p. 164). During the experiments on the time-course of lysis centrifugation was not used and there was a delay of 30 minutes before significant lysis was observed - this could be due to the delay in settling of RBC and effector cells in the culture wells. The kinetics of lysis observed are similar to that seen with K-cell lysis of chicken RBC (Wisloff et al 1974, Calder et al 1974) and lymphoblastoid cells (Trinchieri et al 1977) where there is a gradual increase in lysis of several hours up to 18-24 hours. In contrast, monocyte-mediated lysis is usually rapid and complete within 6 hours (Kovithavongs et al 1975). These differences suggest that a K-cell is involved in the D-anti-D system.

Further evidence for direct cell-to-cell contact is obtained in para. 4.0, p. 172 where D-negative RBC were not lysed even though intimately mixed with D-positive RBC which were being lysed ie. there was no "innocent bystander" effect. This also suggests that the lytic process must either be extremely short-range or decay very rapidly. Transfer experiments failed to demonstrate the presence of any supernatant lytic factor.

Although intimate cell contact is required for lysis of RBC, phagocytosis is not necessary. Visual examination of cell cultures at various times failed to reveal the presence of phagocytosis - in fact the anti-D used to sensitise RBC for lysis did not even cause obvious cell-to-cell contact in the form of rosettes, even in the strongest concentrations used. Removal of phagocytic cells did not impair RBC lysis, and indirect measurement of phagocytosis by intracellular ^{51}Cr failed to reveal signficiant phagocytosis of sensitised RBC (para. 3.0, p. 169).

Some degree of cell mobility would appear to be necessary however, to account for the number of RBC lysed per effector cell. Most estimates of K-cell numbers are of the order of 1-10% of mononuclear cells, (Biberfield et al 1975) and in section 5.0, p. 176 it was calculated that up to 40 RBC could be lysed per K-cell. Some degree of motility is necessary to achieve this in the culture conditions used. Further evidence for the requirement of motility and intact microfilament function was obtained from the inhibition experiments with cytochalasin B (section 7.0, p. 193). Some degree of "gripping" of the target cell may be necessary to achieve the appropriate degree of contact as shown previously (Roitt et al 1976). The divalent cations Mg^{2+} and Ca^{2+} are not required for rosette formation between IgG sensitised RBC and mononuclear cells (Lay & Nussenzweig 1969) and have also been shown to be unnecessary for K-cell recognition of the target (Goldstein & Smith 1976). However, these cations are necessary for lysis to be induced. It has been shown that Mg^{2+} is required for sheep RBC lysis and

Ca^{2+} for Chang cell lysis (Goldstein & Fewtrell 1975). With the D-anti-D system, it appears that Ca^{2+} is required, since Mg^{2+} alone is insufficient (para. 8, p. 200) to support K-cell lysis. From the experiments done, one cannot say whether both Ca^{2+} and Mg^{2+} are required or whether Ca^{2+} alone is sufficient. Further experiments are required to define the cation requirements in more detail at the stage in lysis at which they act. The differences in cation requirements with the present ADCC system may be related to the effector cells active - the sheep RBC is more susceptible to lysis by the monocytic cell whereas the Chang cell (and the papainised human RBC) is more susceptible to lysis by the lymphoid K-cell. It is not clear why these cations are required. Their presence is necessary for phagocytosis, but this is not an essential pre-requisite for ADCC lysis of targets. It may be that they are required to trigger intracellular events analogous to the secretion of proteins from cells or lymphocyte transformation (Goldstein & Gomperts 1975).

Indirect evidence for the nature of the lytic process is obtained from the use of metabolic inhibitors (para. 7.0, p. 182). It was shown that a metabolically active cell is necessary for maximal lysis. An intact microtubule system is also required and stabilisation of lysosomal membranes by hydrocortisone also inhibits lysis. It appears reasonable to postulate that once the K-cell has been activated by contact with an appropriate target cell coated with antibody, the synthesis of a lytic protein factor takes place and that this is then transported to the cell exterior via the microtubule system as lysosomal material. This lytic factor is probably

very labile since bystander RBC are not lysed and supernatants from lytic cultures were inactive - in contrast to the lymphocytotoxins produced by mitogens stimulated T-lymphocytes (Hiserodt & Granger 1976). The lability may partially explain why the effector cells do not lyse their own cell membranes since the lymphoid cell surface membrane is not intrinsically resistant to ADCC lysis (Rachelefsky et al 1975, Kovithavongs et al 1974).

The experiments described do not distinguish between the various stages of lysis ie. recognition, lethal hit, and target cell disintegration. In order to determine at which stage the divalent cations are required, and the various inhibitors take effect, it would be necessary to carry out further work with time course experiments. Direct examination of the target-effector interaction by a variety of microscopic methods (eg. time-lapse photography, scanning electron microscopy, transmission E-M) would also yield a great deal of useful information.

The nature of the trigger to lysis is unknown but it has been reported that the activated Fc portion of cell bound IgG is an essential factor (para. 2.3, p. 22) when the Fc portion is "activated", stronger effector cell binding results and this is normally achieved when the antibody binds to the appropriate target cell antigen via the Fab part. The weak binding of native anti-D IgG was demonstrated in para. 6.0, p. 179, where free anti-D did not bind to the effector cells during attempts to "presensitise" the K-cell by a cytophilic effect. In contrast, presensitised target cells are very efficiently lysed. It has been reported by other workers (Greenberg & Shen 1973) that monocytic effector

cells can be "armed" or activated to lyse specific target cells by an appropriate combination of antigen/antibody complex, whereas the native antibody is ineffective. This is presumably due to the effect that the Fc determinant of complexed antibody binds more firmly to the Fc receptor of the effector cell.

It has not been formally shown in the present series of experiments that the Fc portion of IgG anti-D is required for lysis since F(ab)2 fragments should be tested for lytic potential, and this was not done. However, indirect evidence is obtained from the very efficient inhibition of anti-D induced RBC lysis by heat aggregated IgG and high concentrations of native IgG (para. 9.0, p. 203). Although there is no doubt that the active component of the anti-D sera used was IgG, it has not been shown that IgM is inactive, due to lack of suitable material although this has been done by many other authors (eg. MacLennan 1972, Perlmann 1972) for other ADCC systems, and also with a D-anti-D system (Dr. Fleer, Central Laboratory, Amsterdam - personal communication).

Indirect evidence for the role of IgG is obtained from the inhibition experiments in para. 9.0, p. 203 where only IgG was effective in inhibiting K-cell lysis; IgA and IgM at concentrations above that normally found in the plasma, were not inhibitory. The inference is that the K-cell receptor is specific for the Fc portion of IgG, and not IgA or IgM. Aggregated IgG has "activated" Fc regions and has been shown to bind more firmly to Fc receptor of mononuclear cells than monomeric IgG (Dickler & Kunkel 1972). Aggregated IgG should therefore inhibit more efficiently than an equivalent amount of native IgG and this was shown in para. 9.2,

p. 208.

The high affinity of aggregated IgG for the K-cell Fc receptors was convincingly shown in experiments where preincubation of mononuclear cell suspensions with aggregated IgG abolished the lytic activity when these cells were subsequently transfered to ADCC assays (fig. 9.2.1, p. 209). Similar results have been reported for other K-cell systems (Perlmann 1972, MacLennan 1972).

The anti-D antibody used to induce lysis was known to be almost entirely of IgG_1 subclass (chapt. III, p. 67). This is of interest, because it has been shown in other systems that IgG_1 and IgG_3 antibodies are the most efficient at inducing lysis and that these are the most efficient at fixing complement (Abramson & Schur 1972). When the inhibition pattern of IgG subclasses was investigated (para. 9.0, p. 203) there was only minor inhibition with unaggregated material (IgG_1 and IgG_2). After heat aggregation IgG_1 and IgG_3 were unequivocally effective in inhibition, with IgG_1 being better than IgG_3, (table 9.3.2, p. 213). The most efficient inhibition is therefore seen with IgG of the same subclass as the lysis-inducing antibody. The results suggest that there is some degree of cross-reactivity between IgG_1 and IgG_3 in blocking the K-cell Fc receptor. Similar findings have been reported for other ADCC systems (Holm 1974, Larsson et al 1975). The Fc receptor of the K-cell is therefore specific for IgG especially the subclasses IgG_1 and IgG_3, and it is likely that contact with the appropriate activated Fc region of target cells bound antibody is the stimulus to lysis.

5.0 NATURE OF THE EFFECTOR CELL

The problems of conflicting reports in the literature are discussed in chapt. I. The main emphasis of the present work was to identify a non-monocytic effector cell - the so called K-cell. The majority of experiments were therefore carried out with cultures where monocytes had been removed - usually by an adherence method. By the use of plastic dishes and nylon wool columns it was possible to reduce monocytes from some 15-20% to less than 1% on most occasions. It was of some interest to note that morphological methods of assessing monocytes was inaccurate and the use of the non-specific esterase stain demonstrated that mononuclear cell suspensions obtained from Ficoll-Triosil usually contained in excess of 20% monocytes and that following nylon wool adherence less than 1% non-specific esterase positive cells remained. As a result of monocyte removal, the % specific lysis in culture was significantly enhanced (table 2.3.1, p. 230). Furthermore in some experiments the nylon-adherent cells were recovered and these were shown to be inactive in lysing anti-D coated RBC even though many monocytes were present. Several other pieces of evidence suggest that monocytes are not the primary active lytic cell in the ADCC system developed using D-anti-D, with papainised red cells. The dose-response curves obtained by altering the E/T ratio at a fixed anti-D concentration are very similar before and after monocyte removal (fig. 5.2.1, p. 125) with a suggestion of increased activity post nylon. It was also shown that the dose-response curves obtained by diluting anti-D at a fixed E/T ratio indicated enhanced lysis following monocyte removal (fig. 6.2.1 and 6.2.2, p. 138). Phagocytosis was not necessary for lysis to occur. The results obtained with cytochalasin B pre and post monocyte removal

suggested that there were at least 2 types of lytic activity present - one manifest as a result of stimulation of lysosomal enzyme release only seen in the presence of monocytes, the other manifest by dose-dependent inhibition by cytochalasin B. Removal of phagocytic monocytes following carbonyl iron ingestion did not reduce ADCC activity in cultures (fig. 3.0, p. 234) indicating that a population of phagocytic but non-adherent monocytes was not responsible for RBC lysis. This was not unexpected since phagocytic monocytes (estimated by latex particle ingestion) were not seen after nylon column passage.

The bulk of the evidence therefore suggests that in the ADCC assay developed, the mature, adherent and phagocytic monocyte as demonstrated by non-specific esterase staining is not the effector cell, contrary to what has been shown for other systems using human RBC (Holm et al 1974, Poplack et al 1976). It is not clear why these differences exist, but there are three possible reasons. Firstly, high concentrations of human serum are used in culture compared with other experimental systems described (minimum 10% and more usually 40% human serum). Since monocyte mediated functions (including ADCC activity) are extremely sensitive to the presence of human IgG, the high serum concentration used may be inhibitory. Secondly, the RBC are papainised. The cell surface may be altered in such a way as to make the K-cell lysis more effective than monocyte lysis and there is some evidence that with untreated human RBC, only the monocyte is active (Zeijlemaker et al 1975). However, Holm (1972) used trypsinised human RBC and found monocytes more active than lymphoid K-cells after the use of this proteolytic enzyme; thus the relative importance of the papainisation

of RBC is unclear. The third possibility is that there is something unique about the anti-D used to initiate lysis. This is unlikely since similar effective lysis was obtained with a variety of different anti-Ds obtained from other sources. However, it is of interest that RBCs maximally sensitised with the Louden anti-D did not form rosettes with monocyte-rich suspensions (Dr. A. Fleer, Central Laboratory, Amsterdam - personal communication).

It is likely that in the assay system both monocytes and a lymphoid K-cell are effective in lysing human RBC and that removing the monocytes reveals the lytic potential of the effector K-cell. The preliminary results obtained with the specific antimonocyte serum may have resolved the above dilemma. Despite being depleted of mature monocytes with adherent and phagocytic properties, a significant number of cells remain with a monocyte surface marker. Furthermore the antimonocyte serum inhibits the ADCC activity of the post nylon cell population (para 7.0, p. 248). It is possible therefore that the so-called lymphoid K-cells are in fact monocyte precursors, or a subset of monocytes which do not have phagocytic and adherence characteristics, but possess monocyte-specific surface markers. Further investigations with the antimonocyte serum are necessary to validate the hypothesis and deserve high priority in view of the general confusion as to the origin of the effector K-cell with the morphological characteristics of a lymphocytic cell.

Various surface marker studies on the effector cell population before and after nylon column passage showed that the active effector population was depleted of surface immunoglobulin-bearing cells but not C3 or Fc receptor-bearing cells. (para. 2.2, p. 227). This suggests that the effector cell

does not have an SIg receptor but does have C3 and Fc receptors (see para 2.2, p. 11). This was further investigated in a series of depletion experiments with rosetting and RBC monolayers. Although technical difficulties were encountered, it was concluded that the effector cell did not have the T lymphocyte sheep RBC marker but did have a C3 and an Fc receptor as noted in other ADCC systems (see chapt. I). The RBC monolayer studies were of interest because after prolonged incubation, plaques, or zones of lysis, could be observed around certain cells in the presence of anti-D sensitised monolayers. The mechanism of lysis could also be profitably investigated using these monolayers since direct visualisation is relatively straightforward.

6.0 APPLICATIONS OF THE K-CELL ASSAY

The attempts to use the K-cell system to determine the D-antigen density of RBC, and hence the zygosity, have already been discussed above. Because of the increased sensitivity of the K-cell assay over conventional serological techniques, its application deserves further study since appropriate adjustments of the culture variables may eventually produce a suitable technique for the determination of D-antigen zygosity. This test would then be a useful aid for determining the Rhesus genotypes of RBC where family studies were not possible.

An inherent problem with using the K-cell assay for any form of comparitive studies is the biological variation seen between individuals. It is important to define any broad differences between categories of normal individuals (eg. on the basis of age or sex) before applying this test

to the study of disease. As a result of the preliminary studies carried out (para 3.0, p.272) it is possible to infer that during health, any one individual has a level of K-cell activity which remains relatively constant; "good" K-cell donors expressed high lytic potential whereas "poor" K-cell donors expressed low lytic potential. Nevertheless, sudden fluctuations were observed from time to time in all individuals studied which could not be ascribed to any external event.

The weakness of the data presented for the individual donors is that only one E/T ratio (10:1) is available. As discussed previously it would be better to express K-cell activity in terms of the 50% specific lysis point; however this information was not available on all the individuals over a long period of study. It is possible that the results expressed this way would be more "stable" and show fewer inexplicable fluctuations. It may also be possible to "categorise" individuals better in terms of K-cell capacity. It would also be valuable to quantify the actual number of K-cells present in a given amount of blood by the monolayer plaque method since one would be able to correlate functional activity (lytic potential) with numbers of K-cells. This information should be available for normal individuals before any meaningful interpretation can be made of the findings in various disease categories eg. autoimmune haemolytic anaemia.

Interesting results were obtained using cord blood mononuclear cells. Good ADCC activity was shown when monocyte-depleted cultures were used, but poor activity when monocytes remained. Part of the explanation may be that

there is relative enrichment of the cord blood K-cells following monocyte depletion. The presence of neonatal K-cells with the ability to lyse Chang cells has been shown by Campbell et al (1974) and the present preliminary results show that RBC targets can also be lysed. The presence of the K-cells in the neonate lends weight to the hypothesis that at least part of the RBC damage seen in Haemolytic Disease of the Newborn (H.D.N.) is due to K-cell action in lysing antibody-coated foetal RBC. It is necessary to carry out further experiments to validate this hypothesis, in particular, to show that foetal RBC are susceptible to K-cell lysis and that foetal K-cells will lyse autologous RBC. By analogy with the adult it is likely that this is the case.

It is important to be able to predict the potential severity of H.D.N. so that appropriate action may be anticipated. Present methods of assessing antibody levels rely entirely on agglutination characteristics of the antibody whether estimated manually, or by the Autoanalyser. With the K-cell assay it is possible to control the variables in such a way that antibody is measured in terms of specific lysis ie. biological activity relevant to the clinical situation. The preliminary experiments reported in para. 2.0, p.266 attempt to assess whether there is any correlation between agglutination activity and specific lysis for several sources of anti-D. Although some methodological problems were encountered it does appear that there need not be any correlation between anti-D titre and anti-D induced specific lysis, and that each source of anti-D behaved somewhat differently. It would therefore be important to extend these studies to antibodies where the clinical

history eg. in terms of H.D.N. is known and to see whether there is better correlation of K-cell specific lysis with severity than conventional quantitation.

If this is found to be so, then an explanation is required. It may be that the IgG subclass distribution of anti-D is important in determining the severity of H.D.N. since IgG_1 and IgG_3 have greater lytic potential. Further studies are therefore required to correlate the lytic activity with IgG subclass distribution.

Some insight into the "physiology" of the K-cell is gained from the results seen following adrenalin infusion and D-positive RBC infusion into healthy normal volunteers. The literature relating to previous work is reviewed in para. 5.0, p.278 but the main feature is an increase in absolute numbers of circulating B cells and K-cells some 15-30 minutes after adrenalin infusion or exercise. The very preliminary results obtained with one infusion were in agreement with other work reported. There was an increase in specific lysis of RBC at 15 minutes which coincided with an increase in the absolute number of C3 receptor bearing cells. Most of these are B lymphocytes, but some will be the K-cells which also have a C3 receptor. The increased specific lysis in this case is therefore likely to be due to an increase in numbers of K-cells rather than increased functional capacity. In vitro experiments would be necessary to determine whether adrenalin also increased the lytic capacity of a given number of cells. This increase in K-cell activity with adrenalin could be important in times of stress to increase the defence mechanisms eg. during infection. On the other hand this

increase could on occasions be detrimental, eg. during a haemolytic transfusion reaction, or in a patient with autoimmune haemolytic anaemia.

Alterations in K-cell activity were also seen following the infusion of D-positive RBC into Rh-ve male volunteers. In this instance, a fall in K-cell activity was seen following infusion. Anti-D was not present at the time of infusion so that active haemolysis was not observed. Nevertheless, there was a transient reduction in K-cell activity at 12-14 days, followed some time later by greatly increased K-cell activity. It is possible that there is a redistribution of K-cells following infusion, eg. to the spleen. If the K-cells are in fact monocyte precursors, then the stimulus of the D-positive RBC may in some way enhance their development to mature monocytes/ macrophages in the RES where they may "clear" the D positive RBC more quickly from the circulation.

Although the changes in K-cell activity was seen in each of 3 volunteers studied, it is important to determine whether this occurs with all such donors, or there is a difference between "responders" and "non-responders" to D-positive RBC (in terms of anti-D production). With such a distinction it may prove possible to identify the responders at an early stage and save considerable time and effort on the part of the donor (and the Blood Transfusion staff).

The enhanced activity after the exposure to D-positive RBC may indicate that the K-cells have some form of "immunological memory". It would be necessary to evaluate 2 or more ADCC systems in parallel, with different target cells, in order to determine whether the increased K-cell

activity seen is specific (for D-anti-D) or general. The latter is more likely since the K-cells apparently lack specificity in terms of target cell lysis. The possibility of enhancing K-cell activity, eg. to tumours, or infectious organisms is potentially important as a means of increasing host resistance. However, a great deal of work remains to be done in order to realise that aim.

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